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(54) PROCEDE AMELIORE DE DETECTION ET D'IDENTIFICATION DE MICRO-ORGANISMES CAUSES D'INFECTIONS
(54) IMPROVED METHOD FOR DETECTING AND IDENTIFYING MICROORGANISM CAUSATIVE OF INFECTION

(57)
A microorganism causative of an infection is quickly and highly sensitively detected and/or identified by obtaining phagocytes from a clinical specimen containing phagocytes originating in a living body, immobilizing the obtained phagocytes, treating the phagocytes so as to enhance the permeability of the phagocyte membrane and expose DNA of the microorganism causative of the infection which is anticipated as being contained in the phagocytes, and then using a detection DNA probe hybridizable with the DNA under stringent conditions.

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(57) Abrégé/Abstract:
A microorganism causative of an infection is quickly and highly sensitively detected and/or identified by obtaining phagocytes from a clinical specimen containing phagocytes originating in a living body, immobilizing the obtained phagocytes, treating the phagocytes so as to enhance the permeability of the phagocyte membrane and expose DNA of the microorganism causative of the infection which is anticipated as being contained in the phagocytes, and then using a detection DNA probe hybridizable with the DNA under stringent conditions.

ABSTRACT

Causative microorganisms of infectious diseases are detected and/or identified rapidly and high-sensitively by taking phagocytes from the clinical specimens containing active phagocytes, immobilizing the phagocytes so taken, treating the phagocytes to improve cell membrane permeabilities thereof, further treating the phagocytes to bare DNA in the causative microorganisms which might be existed in the phagocytes, and detecting the causative microorganisms with DNA probes which can hybridize with such DNA under stringent conditions.

SPECIFICATION

IMPROVED METHOD FOR DETECTING AND IDENTIFYING CAUSATIVE MICROORGANISMS OF INFECTIOUS DISEASES

TECHNICAL FIELD

The present invention relates to an improved method for detecting and identifying causative microorganisms of infectious diseases. The present invention also relates to a kit for detecting and/or identifying causative microorganisms of infectious diseases, a method for monitoring genes from exogenous microorganisms in clinical specimens, and a method for determining causative microorganisms of sepsis and those of bacteriemia.

BACKGROUND ARTS

Although the hemoculture methodologies have popularly been used conventionally as a mean to verify bacteria in the blood, since this methodology needs about from 3 to 14 days to culture and isolate the subjected bacteria and detection rates thereby are as low as about 10%, it was not well contributed in the diagnosis for treating serious diseases like sepsis.

The present inventors had invented, to solve such problems, a method for detecting and identifying exogenous-microorganisms digested with phagocytes comprising a step of detecting genes from such exogenous-microorganisms in the phagocytes by *in situ* hybridization employing a probe which can specifically hybridize with the genes (Japanese Patent Publication No. 7-40).

The method of Japanese Patent Publication No. 7-40 have been in the limelight in the field of infectious deseases because, in comparison with the conventional hemoculture methodology, the method allowed about four times rapidly detection of the subjected bacteria in bloods from patients who are under the suspicion about sepsis, and detection results were appeared within 24 hours.

Objects of the present inventions is an improvement of detection effects and of detection sensitivity to be offered by the method according to Japanese Patent Publication No. 7-40 for detecting and/or identifying causative microorganisms of infectious diseases by taking phagocytes from the clinical specimens containing active phagocytes, immobilizing the phagocytes so taken, treating the phagocytes to improve cell membrane permeabilities thereof, further treating the phagocytes to bare DNA in the causative microorganisms which might be existed in the phagocytes, *in situ* hybridizing DNA so bared with detective DNA probe(s) which can hybridize with such bared DNA under stringent conditions, and detecting and/or identifying the causative microorganisms based on signals so detected.

DISCLOSURE OF INVENTION

The present invention has been completed in view of the problems aforenoted and the merits thereof are as follows.

A method for detecting and/or identifying causative microorganisms of infectious diseases by taking phagocytes from the clinical specimens containing active phagocytes, immobilizing the phagocytes so taken, treating the phagocytes to improve cell membrane permeabilities thereof, further treating the phagocytes to bare DNA in the causative microorganisms which might be existed in the phagocytes, *in situ* hybridizing DNA so bared with detective DNA probe(s) which can hybridize with such bared DNA under stringent conditions, and detecting and/or identifying the causative microorganisms based on signals so detected, the method comprises at least one condition(s) to be selected from the following conditions (1)-(8) of;

(1) Cell density (X cells/ml) of the phagocytes to be immobilized is 5×10^{6} cells/ml$<$X cells/ml$<1\times10^{8}$ cells/ml,

(2) Lysostaphin is applied into the step to bare DNA in the titer of from 1 Unit/ml to 1,000 Units/ml,

(3) Lysozyme is applied into the step to bare DNA in the titer of from 1,000 Units/ml to 1,000,000 Units/ml,

(4) N-acetylmuramidase is applied into the step to bare DNA in the titer of from 10 Units/ml to 10,000 Units/ml,

(5) Zymolyase is applied into the step to bare DNA in the titer of from 50 Units/ml to 500 Units/ml,

(6) Surfactant is applied into the step of *in situ* hybridization,

(7) Such DNA probe(s) is/are one or more DNA probe(s) to be determined with their chain length of from 350 bases to 600 bases, and

(8) Concentration of such DNA probe(s) is from 0.1 ng/μl to 2.2 ng/μl.

The step to bare DNA employs preferably one or more enzyme(s) selected from Lysostaphin in the titer of from about 10 to about 100 Units/ml, Lysozyme in the titer of from about 10,000 to about 100,000 Units/ml, N-acetylmuramidase in the titer of from about 100 to about 1,000 Units/ml and Zymolyase in the titer of from about 100 to about 500 Units/ml.

The step to bare DNA preferably employs enzyme(s), and the enzyme(s) is/are subjected to a reaction to be performed under the temperature of from about 26℃ to about 59℃ for from about 15 to about 120 minutes.

The step to bare DNA further employs preferably subtance(s), in particular, phenylmethylsulfonyl fluoride to keep a form of the phagocytes under the concentration of preferably from about 10μmol/l to about 10mmol/l.

As the subtance to keep a form of the phagocytes, substance dissolved into dimethylsulfoxide is preferable. The substance dissolved in dimethylsulfoxide is employed as that to keep a form of the phagocytes, then, concentration of the dimethylsulfoxide in a solution to be used in the step to bare DNA is adjusted to less than 5%.

The *in situ* hybridization step is perfomed by hybridizing DNA with DNA probe(s) under the precence of surfactant(s), in particular, the anionic surfactant, preferably, sodium dodecyl sulfate (SDS).

Hybridization reaction in the *in situ* hybridization step is performed under the temperature of from about 20℃ to about 50℃ and time of from about 30 to about 900 minutes.

The method further comrises, prior to the immobilization step, a step to mount the phagocytes so taken onto the solid support and a slide coated with 3-aminopropyl triethoxysilane is employed as such solid support.

Pigment(s) is/are also employed at the signal detection to distinguish contrast between signals and cells. Preferably, blood is employed as the clinical specimen.

The present invention further provides a kit for detecting and/or identifying causative microorganisms of infectious diseases by taking phagocytes from the clinical specimens containing active phagocytes, immobilizing the phagocytes so taken, treating the phagocytes to improve cell membrane permeabilities thereof, further treating the phagocytes to bare DNA in the causative microorganisms which might be existed in the phagocytes, *in situ* hybridizing DNA so bared with detective DNA probe(s) which can hybridize with such bared DNA under stringent conditions, and detecting and/or identifying the causative microorganisms based on signals so detected, the kit comprises the following elements (1)-(2) of;

(1) at least one enzyme(s) to be employed in the step to bare DNA which is/are selected from Lysostaphin, Lysozyme, N-acetylmuramidase and Zymolyase; and

(2) at least one detective DNA probe(s).

Then, the present invention further provides a method for monitoring a gene of exogenous microorganisms digested with the phagocytes in the clinical specimens containing active phagocytes comprising the step of detecting the gene with *in situ* hybridization method employed in the foregoing method, wherein the gene of exogenous microorganisms in the clinical specimens is monitored.

The present invention further provides a method for diagnosing sepsis or bacteriemia comprising the step of identifying a gene of candidate causative microorganisms with *in situ* hybridization method employed in the foregoing method, wherein the causative microorganisms for sepsis or bacteriemia are determined based on the identification results.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a view of illustrating results of *in situ* hybridization performed under (a) the absence of surfactant (SDS) and (b) the presence of surfactant (SDS).

Figure 2 is a view of illustrating a manner of leukocytes immobilized with the various cell density.

Figure 3 is a view of illustrating a time-coursely change on lytic enzyme activity against (a) *Staphylococcus aureus* and *Staphylococcus epidermidis*, (b) *Pseudomonas aeruginosa* and *Escherichia coli*, and (c) *Enterococcus faecalis*.

Figure 4 is a view of illustrating concentration-dependency effects by addition of DMSO against lytic activity to be offered by (a) 300 Units/ml of N-acetylmuramidase, (b) 10,000 Units/ml of Lysozyme, and (c) 50 Units/ml of Lysostaphin.

Figure 5 is a view of illustrating results on addition of (a) protease 0.2 Units/ml only, (b) PMSF 1 μmol/ml, (c) PMSF 10 μmol/ml, (d) PMSF 0.1mmol/ml, and (e)PMSF 1 mmol/ml to study effects of PMSF to be used to suppress the function of protease

which changes a morphological form of leukocyte.

Figure 6 is a view of illustrating that, in the phagocytosis samples prepared according to the present invention, phagocytes digested bacteria and morphological forms of such digested bacteria were changed.

Figure 7 is a view of illustrating effects of enzyme-treatment in the phagocytosis samples and manner of the phagocytosis samples containing (a) *Staphylococcus aureus* prior to the treatment, (b) *Enterococcus faecalis* prior to the treatment, (c) *Staphylococcus aureus* of (a) with the treatment, and (d) *Enterococcus faecalis* of (b) with the treatment.

Figure 8 is a shematic view of the slide on which the phagocytosis samples were smeared to study optimum probe concentration under the *in situ* hybridization.

Figure 9 is a shematic view of the slide on which the phagocytosis samples were smeared to study optimum temperature under the *in situ* hybridization.

Figure 10 is a view of illustrating the signals appeared in the resluts of Southern Blot (the upper row) and Electrophoresis (the lower row) together with the length of the labelled detective probes prepared by putting digoxigenin-labels on (a) SA probes and (b) PA probes.

Figure 11 is a view of illustrating the signals detected by *in situ* hybridiozation of the digested *Escherichia coli* with detective probes of (a) EC-24、(b) EC-34、(c) EC-39 and (d) the mixed probes (MIX) of the foregoing probes (a)-(c).

BEST MODE FOR CARRYING OUT THE INVENTION

Any clinical specimen containing active phagocytes is applicable as a specimen to be employed in the present embodiment and may includes fluidus like blood, histofluid, lymph, neurolymph, pus, pituita, nasal mucus or sputum. Then, active phagocytes are contained in urea, ascites or dialysate in the disorder like diabetes, nephropathia or hepatopathy and are also remained in the lotion used to wash rhinonem, bronchus, skin, various organs or bones, they can therefore also be employed as specimen of the present invention.

In addition thereto, tissues taken from skin, lung, kidney, mucosa or the like are also employed as the clinical specimen in the present invention. This is because that macrophage, which is one of phagocytes, has various forms including monocytes, alveolar macrophage, celiac macrophage, fixed macrophage, free macrophage, Hansemann`s macrophage, inflammatory macrophage, liver Kupffer cells and brain microglia cells, tissues containing those can therefore also be employed, besides blood, as the clinical specimen of the present invention. For example, causative microorganisms of nephritis can be detected and identified by collecting kidney tissues through biopsy from patients who are under the suspicion about nephritis, taking phagocytes in the tissues through digestion thereof with enzymes like trypsin to exfoliate cells, and utilizing the phagocytes so taken.

The term `phagocytes` used herein is directed to any cell which can incorporate into itself foreign objects like exogenous microorganisms and may includes, for example, macrophage, monocytes, neutrophil and eosinophil. Phagocytes line like U937 Cell, HL60 Cell or the like is also available. Exogenous microorganisms which may cause infectious diseases are microorganisms to be digested with phagocytes and may includes, for example, bacteria, mycete, virus, protozoon, parasite or the like. Bacteria may include, for example, *Staphylococcus*, *Pseudomonas*, *Enterococcus*, *Colibacillus*, *Streptococcus*,

Pneumococcus, *Tubercle bacillus*, *Helicobacter pylori*, *Listeria*, *Yersinia*, *Brucellar* or the like. Mycete may include, for example, *Candida*, *Aspergillus*, *Actinomyces*, *Coccidioides*, *Blastomyces* or the like. Virus may include Influenza virus, Poliovirus, Herpes virus, Hepatitis virus and AIDS virus. Protozoon may include, for example, Karyamoebina falcata, Trichomonas vaginalis, Malaria, Toxoplasma or the like. Parasite may include, for example, Trypanosoma or the like. In particular, the causative microorganisms of sepsis or bacteriemia may include, for example, Gram-Positive Bacteria of *Staphylococcus* genus (*Staphylococcus aureus*, *Staphylococcus epidermidis*) and *Enterococcus* genus (*Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptociccus agalactiae*), Gram-Negative Bacteria like *Colibacillus*-related *Enterobacteriaceae* Family of *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae* (*Klebsiella oxytoca*, *Serratia marcesens*, *Proteus vulgaris*, *Citrobacter freundii*), aerophilic rod of *Pseudomonas* genus (*Pseudomonas aeruginosa*), anaerobe of *Clostridium* genus (*Clostridium perfringens*), *Bacteroides* genus (Bacteroides fragilis) or the like. Also, *Acinetobacter calcoaceticus*、*Aeromonas hydrophilia*、*Flavobacterium meningosepticum*、*Bacillus cereus* can rarely be fallen within the causative microorganisms.

Phagocytes (Leukocytes) fractions can be taken from the clinical apecimen according to the conventional method. For example, about 5ml (or 10ml with a few leukocyte) of heparinized venous bloods were obtained and the bloods were mixed with the blood components separative reagent (adjusted with sterilized-purified water to be 25ml as their final volume containing 225mg of Sodium Chloride and 1.5 g of Dextran (Molecular Weight: 200,000-300,000)) in the ratio of 4:1. Leukocyte fractions (the upper layer) were then obtained by leaving them at from about 10℃ to about 40℃ for from about 15 minutes to about 120 minutes, preferably, about 37℃ for about 30 minutes. Leukocytes were appeared by centrifuging the leukocyte fractions so taken in from about 100×g to about 500

×g, at from 0℃ to about 20℃ for about from 3 minutes to about 60 minutes, preferably, in from about 140×g to about 180×g, at about 4℃ for about 10 minutes. Hemolysis is preferable if erythroblasts are entered at this step. Pelletized leukocytes so obtained were, for example, suspended with 1 ml of sterilized-purified water and the suspension were immdeditely put into an isotonic state by adding thereto excessive amounts of PBS (prepared by diluting twenty-fold with sterilized-purified water the raw solution (PBS Raw Solution; hereinafter simply referred to as `PBS Raw Solution`) which have been adjusted with sterilized-purified water to be 120ml as their final volume containing 18.24 g of Sodium Chloride, 6.012 g of Sodium Monohydrogen Phosphate Didecahydrate and 1.123 g of Sodium Dihydrogen Phosphate Dihydrate), and such suspension were re-centrifuged in from about 140×g to about 180×g, at 4 ℃ and for about 10 minutes. Otherwise, such digested phagocytes can be adhered to the slides to be noted later through their native adhesionability without any centrifugation aforenoted.

Methodology to fix the leukocytes may include, for example, Carnoy s fixation. In particular, leukocytes are mounted onto a support (a supportive medium) which can hold the leukocytes and are immersed in Carnoy s fixative solution (prepared by mixing Ethanol, Chloroform and Acetic Acid in the volume ratio of 6:3:1) for 20 minutes and were immersed in from about 50% to about 90%, preferably, about 75% Ethanol solution for five minutes. Finally, they were completely air-dried.

Insoluble materials are preferable for the support and may include, for example, glass, metal, synthesized resin (e.g., polystyrene, polyethylene, polypropylene, polyvinyl chloride, polyester, polyacrylic ester, nylon, polyacetal and fluoric resin) and polysaccharide (e.g., cellulose and agarose).

A form of the insoluble support can be changed optionally and may include, for example, that of plate, tray, ball, fiber, rod, disk, container, cell or tube.

Particularly preffered support in the present embodiment is to emply slides. Such slide may include, for example, the slide (PRODUCTS ID. S311BL) of JAPAN AR BROWN CO., LTD. This slide (PRODUCTS ID. S311BL) has 14 circular wells of 5mm diameter. In order to improve an adhesionability of the subjected cells at the actual use, APS coated slides to be made by coating 3-aminopropyltriethoxysilane (APS, SIGMA) onto the slides are recommended. The other silides to be made by coating poly-L-lysine or gelatin are also available.

APS coated slides are prepared by putting the slides (PRODUCTS ID. S311BL) onto a holder, immersing them into the diluted neutral detergent for 30 minutes or more, removing well the detergent with tap water, then washing the slides with purified water, and drying well the same under the higher temperature (100℃ or more) followed by leaving it under the room temperature. Thereafter, these slides are immersed into acetone containing 2% APS for one minute and are immediately rinsed gently with acetone then with sterilized-purified water. These slides were then air-dried. These slides are re-immersed into acetone containing from about 1% to about 10% APS for one minute, are immediately rinsed gently with acetone then sterilized-purified water, and are air-dried. Finally, these slides are dried under the temperature of from about 20℃ to about 60℃, preferably of 42℃ to realize the APS coated slides.

Leukocytes are mounted onto the APS coated slides preferably by smearing the leukocytes to realize the extended mono-layer thereof and drying the same. Cell population (x cells/ml) of the immobilized phagocytes should be adjusted to that of about 5×10^{6} cells/ml$<$ x cells/ml$<$about 1×10^{8} cells/ml, preferably, about 1×10^{7} cells/ml$\leqq$ x cells/ml$\leqq$about 5×10^{7} cells/ml.

Then, according to change on the phagocytes population per 1ml, leukocyte population to be immobilized in the single well of the APS coated slide (y cells/well (diameter: 5mm)) are

adjusted that of about 2.5×10^4 cells/well$<$y cells/well$<$about 5×10^5 cells/well, preferably, about 5×10^4 cells/well$\leqq$y cells/well$\leqq$ about 2.5×10^5 cells/well. In particular, pelletized leukocytes are prepared by centrifuging leukocyte fractions in from about 140$\times$g to about 180$\times$g, at 4 ℃ and for 10 minutes, adding small amount of PBS to the pelletized leukocytes, suspending the same, and counting the population with a hemacytometer. Leukocytes were duly mounted on the APS coated slides by smearing 5μl of the leukocyte suspension into each well of the slides adjusted with PBS to be cell population of from about 5×10^4cells/well to about 2.5×10^5 cells/well, then extending mono-layer of the leukocytes, and completely air-drying the same.

In order to accelerate permeability of the phagocyte membrane, they were immersed in PBS for from about 3 minutes to about 30 minutes, then in the solution prepared by diluting from about 2-fold to about 50-fold a pretreatment reagent (prepared by mixing 1.25g of Saponin, 1.25 ml t-octylphenoxy-polyethoxyethanol (specific gravity of 1.068-1.075 (20/4℃), pH (5w/v%) 5.5-7.5) and 25ml PBS Raw Solution, and adjusting with sterilized- purified water to be 50ml as their final volume), and were applied to a centrifuge for from about 3 minutes to about 30 minutes.

In order to bare DNA in the causative microorganisms, an enzyme solution was prepared by adding, per single slide, 1 ml of a reagent solvent (prepared by diluting about 100-fold, with PBS, Dimethyl Sulfoxide (DMSO) containing 0.1 mol/l Phenylmethyl Sulfonylfluoride (PMSF)) to an enzyme reagent (N-acetyl-muramidase, Lysozyme and/or Lysostaphin), then 1 ml of which were dropped under the temperature of from about 20℃ to about 60℃, preferably, from about 37℃ to about 42℃ in the wet chamber onto the area where the leukocytes were smeared and were left for from about 10 minutes to about 60 minutes. Then, they were immersed in PBS containing 0.2 mol/l Hydrochloric Acid (prepared by adding hydrochloric acid to PBS Raw Solution, diluting 20-fold

the same with sterilized-purified water and adujusting final concentration of Hydrochloric Acid to 0.2 mol/l), and were applied to a centrifuge for 3-30 minutes to accelerate thier permeability. 5% or more of DMSO concentration may lower activities of Lysozyme and Lysostaphin, DMSO concentration of less than 5% is therefore preferable. Besides PMSF, the known protease inhibitors like tosyllysinechlromethylketone (TLCK) and a combination thereof are also applicable to keep the form of phagocytes. Solvents like DMSO can be changed optionally to employ such known protease inhibitors.

With regard to the preferable titer range on each enzyme employed in the enzyme reagent, although Lysostaphin offer the substantial effect at the titer of 1 Unit/ml in the lysis of *Staphylococcus aureus*, that of 10 Units/ml or more was necessary in the lysis of *Staphylococcus epidermidis*. Optimum titer on Lysostaphin should therefore be adjusted to from about 1 Unit/ml to about 1,000 Units/ml, preferably, from about 10 Units/ml to about 100 Units/ml. When the titer of Lysozyme was about 10,000 Units/ml, there was no lysis on *Enterococcus faecalis* with N-acetylmuramidase of about 10 Units/ml or less titer. Also, when the titer of N-acetylmuramidase was about 100 Units/ml, there was no lysis with Lysozyme of about 1,000 Units/ml or less titer. Accordingly, optimum titer on N-acetylmuramidase should be adjusted to from about 10 Units/ml to about 10,000 Units/ml, preferably, from about 100 Units/ml to about 1,000 Units/ml, while that on Lysozyme acetylmuramidase should be adjusted to from about 1,000 Units/ml to about 1,000,000 Units/ml, preferably, from about 10,000 Units/ml to about 100,000 Units/ml. When the causative microorganisms are mycete like *Candida albicans*, titer on Zymolase should be adjusted to from about 50 Units/ml to about 500 Units/ml, preferably, from about 100 Units/ml to about 500 Units/ml. In particular, PMSF or the known protease inhibitors are useful in combination with Zymolase.

Enzyme(s) could be selected based on the components difference between Gram-Positive Bacteria and Gram-Negative Bacteria, namely, the difference of peptideglycan or of lipopolysaccharides. Two kinds or more of enzymes are particularly preferable to effectively lyse both Gram-Positive Bacteria and Gram-Negative Bacteria. It was demonstrated in the present invention that the lysis activities offered by the mixed enzymes of Lysozyme, Lysostaphin and N-acetylmuramidase were enhanced in comparison with those by the single enzyme.

Treatment temperature with enzymes on *Staphylococcus aureus* should preferably be adjusted to from about 4℃ to about 60℃, then that on *Staphylococcus epidermidis* should be adjusted to about 25℃ or more, preferably, to about 37℃ or more, and that on *Enterococcus faecalis* should be adjusted to from about 25℃ to less than about 60℃, preferably, from about 37℃ to about 42℃. Accordingly, it is most preferable to designate the temperature range of from about 37℃ to about 42℃ as the optimum treatment temperature. Further, the critical temperature range to be shared with the three bacterial species is expected to the range of from about 26℃ to about 59℃.

Then, treatment time with enzymes on any digested sample from *Staphylococcus aureus*、*Staphylococcus epidermidis*、*Enterococcus faecalis* is 20 minutes or more (both at zero minute and 10 minutes were not appropriate), then, since there was no leukocyte in the bacteria, the treatment time should be adjusted to at least about 15 minutes or more, preferably, to about 20 minutes or more, and be designated the time range of from about 30 minutes to about 60 minutes as the optimum treatment time. Treatment time of from about 15 minutes to about 120 minutes may also be applicable.

Then, N-acetylmuramidase is an enzyme which lower an absorbance at 600nm under the reaction at 37℃ for 5 minutes of N-acetylmuramidase with heat-treated dry-powder of *Enterococcus faecalis* in 5mmol/l Tris-HCl Buffer Solution (pH

6.0) containing 2 mmol/l magnesium chloride. Then, when 1 Unit of enzyme activity is defined as an activity to lyse one ug of heat-treated dry-powder of *Streptococcus salivarius* (IFO 3350) in one minute which was determined thereon at 37℃ and pH 7.0, the enzyme of 2,000 Units/mg or more is preferable.

Lysozyme is an enzyme which lower an absorbance at 600nm under the reaction of *Micrococcus luteus* with Lysozyme in PBS at 37℃ for 5 minutes. Then, when 1 Unit of enzyme activity is defined as an activity to lower 0.001 of an absorbance at 540nm in one minute which was determined on *Micrococcus luteus* at 35℃ and pH 6.2, an enzyme of 50,000 Units/mg or more is preferable.

Lysostaphin is an enzyme which lower an absorbance at 600nm under the reaction of *Staphylococcus epidermidis* with Lysostaphin in PBS at 37℃ for 5 minutes. When 1 Unit of enzyme activity is defined as an activity to lower an absorbance at 620nm of 0.240 to 0.125 in 10 minutes which was determined on *Staphylococcus aureus* at 37℃ and pH 7.5, an enzyme of 500 Units/mg or more is preferable.

Zymolase (Products Name: Zymolyase (SEIKAGAKU CORPORATION)) is an enzyme taken from the liquid culture medium of *Arthrobacter lutesul* and have strong degradation activities on cell walls of the active yeast cells. Essential enzymes contained in Zymolase and involved with cell wall degradation is β-1,3-glucan lanimaripentaohydrolase which acts on glucose polymer with β-1,3-bonds and produces laminaripentaose as a main product. Zymolyase-100T is purified in ammonium sulfate fractionation, then in affinity chromatography (Kitamura, K. et al., *J. Ferment. Technol.*, **60**, 257, 1982) and have activity of 100,000 Units/g. However, it is well known that activities of the subjected enzyme are changed according to the kinds of yeast to be substrates, culture condition and growth phase thereof (Kitamura, K. et al., *J. Gen. Appl. Microbiol.*, **20**, 323, 1974; Kitamura, K. et al., *Agric. Biol. Chem.*, **45**, 1761, 1981; Kitamura, K. et al., *Agric. Biol. Chem.*, **46**, 553, 1982).

Zymolyase-100T contains about 1.0×10^7 Units/g of β-1,3-glucanase, about 1.7×10^4 Units/g of protease and about 6.0×10^4 Units/g of mannase, but does not contain any DNAse and RNAse (Kitamura, K. et al. ; *J. Gen. Appl. Micro-biol.*, **18**, 57, 1972). Then, the optimum pH of Zymolyase is from about 5.5 to about 8.5, preferably, from about 6.5 to about 7.5, while the optimum temperature thereof is from about 25℃ to about 55℃, preferably, from about 35℃ to about 45℃. Further, lytic spectrum (genus) to yeast (cells in logarithmic growth phase) may includes *Ashbya, Candida, Debaryomyces, Eremothecium, Endomyces, Hansenula, Hanseniaspora, Kloekera, Kluyveromyces, Lipomyces, Helschkowia, Pichia, Pullularia, Torulopsis, Saccharomyces, Saccharomycopsis, Saccharomycodes, Schwanniomyces* or the like.

In particular, *Candida* genus may include *Candida albicans, Candida tropicalis, Candida parasilosis, Candida galacta, Candida guilliermondii, Candida krusei, Cryptococcus neoformans*. SH compounds, for example, cysteine, 2-mercapto-ethanol, dithiothreitol can be employed as an activator of these enzymes.

Bacteria belonged to these genus may also be employed in the present invetion. 1 Unit of the enzyme activity is defined as an activity to lower about 30% an absorbance at 800nm of the reaction solution (prepared by adjusting with 1 ml of sterilized-purified water to be 10 ml as their final volume containing 1 ml enzyme solution of 0.05～0.1 mg/ml, 3ml of Brewer`s Yeast Suspension (2 mg dry-weight/ml) as a substrate, and 5 ml of M/15 Phosphoric Acid Buffer Solution (pH 7.5)) in two hours which was determined on Brewer`s Yeast Suspension as a substrate at about 25℃. Zymolyase-100T has an activity of 100,000 Units/g.

With regard to the concentration of PMSF (to be added to keep a form of the leukocytes by protecting them from protease) to be used as a reagent solvent, since 10μmol/l or more of PMSF

concentration was effective and a form of the leukocyte was completely kept at the PMSF concentration of 0.1 mmol/l or more, PMSF concentration should be adjusted to the range of from about 10 μmol/l to about 10 mmol/l, preferably that of from about 0.1 mmol/l to about 1 mmol/l. Similarly, DMSO concentration should also be adjusted to less than 5%, preferebaly 2% or less, and more preferably about 1%. Accordingly, the reagent solvent should preferably be prepared by dulting 100 to 1,000-fold, with PBS, Dimethyl Sulfoxide (DMSO) containing 0.1 mol/l Phenylmethyl Sulfonylfluoride (PMSF).

Acetylation of cell membrane proteins may further be performed after the step to bare DNA in the causative microorganisms. In particular, slides are immersed in the acetylation reagent prepared by adding Acetic Anhydrate to the acetylation raw reagent (adjusted with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 7.46g of Triethanolamine and quantum sufficient of Hydrochloric Acid), diluting from about two-fold to about 50-fold, preferably about 10-fold with sterilized-purified water, and adujusting final concentration of Acetic Anhydride to from 0.1% to 3.0%, preferably 0.8%, and are applied to a centrifuge for from 5 to 30 minutes. After then, they are completely air-dried by immersing them succcecively into 75%, 85% and 98% Ethanol for from two to five minutes respectively.

Alkalization of DNA in the causative microorganisms may further be performed to reshape it into the single strand DNA after the step to acetylate the cell membrane proteins. In particular, slides are immersed for from about two minutes to about five minutes in PBS containing from about 10 mmol/l to about 300 mmol/l Sodium Hydroxide, preferably, about 70 mmol/l Sodium Hydroxide (prepared by adding Sodium Hydroxide to PBS Raw Solution, diluting 20-fold with sterilized-purified water, and adujusting final concentration of Sodium Hydroxide to 70 mmol/l). After then, they were completely air-dried by immersing them succcecively into 75%, 85% and 98% Ethanol for

from two to five minutes respectively.

In order to perform *in situ* hybridization with detective DNA probe which can hybridize under the stringent condition to the naked DNA from the causative microorganisms, probe solution (solution containing the detective DNA probe prepared with probe dilution) is applied to the area to be smeared, and is left for from about one hour to about three hours, preferably, for about two hours in the wet chamber of from about 25℃ to about 50℃, preferably, from about 37℃ to about 42℃.

Then, three stained bottles containing the hybridization detergent (prepared by mixing Hybridization Raw Solution (prepared with sterilized-purified water to be 75ml as their total volume containing 13.15g of Sodium Chloride, 6.615g of Trisodium Citrate Dihydrate) in the ratio of Hybridization Raw Solution : sterilized-purified water : Formamide = 5:45:50) are provided and they are successively immersed therein at from about 35 to about 45℃, preferably, at about42℃ for 10 minutes respectively. They are then immersed in PBS and are applied successively to centrifugation on a centrifuge for from about five to about 30 minutes. In particular, the diluted probe solution contains 600μl of Salmon Sperm DNA, 50μl of 100× Denhurt`s Solution, 500μl of Hybridization Raw Solution, 2250 μl of Formamide and 1000μl of 50% Dextran Sulfate. Probe solution contains preferably 15ng of each detective DNA probes and may be adjusted with the probe dilution to be 50μl as their total volume.

Probe concentration of SA, SE, PA, EF, EK is adjusted to from about 0.6 ng/μl to about 1.8 ng/μl, preferably to from about 0.6 ng/μl to about 1.2 ng/μl. Since result at 0.06 ng/ μl was not acceptable while result at 0.6 ng/μl was acceptable, it is preferably to adjust the concentration to at least 0.1 ng/μl or more. Then, since result at 2.4 ng/μl was not acceptable while result at 1.8 ng/μl was acceptable, it is preferably to adjust the concentration to 2.2 ng/μl or less. Further, the optimum concentration of the positive control and

the negative control are adujusted to concentration of from 0.4 ng/μl to 2.0 ng/μl and from 0.6 ng/μl to 2.0 ng/μl respectively, preferably, that of from 0.6 ng/μl to 1.0 ng/μl for the both control.

Time to perfom hybridization is at least 30 minutes or more, preferably 60 minutes or more, and more preferably 90 minutes or more. Time of from about 120 minutes to about 900 minutes may be designated as optimum hybridization time.

It is preferably to use surfactants like sodium dodecyl sulfate (SDS) in the step of *in situ* hybridization because it may allow enhancement of the detection sensitivity. SDS concentration is preferably 1% or less, more preferably from about 0.1% to about 0.5%, more further preferably about 0.25%. SDS is added to the solution to be employed at the hybridization, otherwise, it may be added in advence to the probe diluent or the probe solution.

It is recommended to employ, as the detective DNA probes, one or more kind(s) of DNA probe(s) having from about 350 to about 600 base length, preferably from about 350 to about 550 base length, because the probes are thereby smoothly incorporated into the phagocytes and they may easily and exactly be contacted with genes of the exogenous microorganisms wherein such probes are incorporated. It is not necessary to fall base length (number of bases) of the subjected within the base length range afotrenoted, but is simply recommended to employ probes having base length distribution to be at least partially overlapped with the base length range aforenoted. These probes are made of single or several (one or more) kind(s) of probes. One or more probe(s) may be plural kinds of probes to be hybridized to the single bacterial species or may be plural kinds of probes to be hybridized to the plural bacterial species one to one, but any restriction would not be imposed as far as one or more kind(s) of probes is/are used.

These probes have preferably DNA fragments containing a sequence not to be hybridized anyway to phagocytes themselves and would not cross-hybridize at all to any gene from the unrelevant bacteria species. Specific probes would be prepared in a short time, for example, with a subtraction method. These probes may be prepared and labelled through the conventional nick translation methodology using non-radioisotopic labelling substances like fluorescein isothiocyanate (FITC), biotin, digoxigenin (digoxigenin(DIG)-11-dUTP) or the like. Strand length of the probes can be adjusted most effectively by changing the amount ratio of DNAseI and DNA ploymerase I respectively to be added at the nick translation reaction. For example, in order to effectively label 2μg of DNA probe (SA-24) and adjust strand length thereof (into base length of from about 350 to about 600) which allow the probes to be effectively *in situ* hybridized to the exogenous microorganisms DNA, in the reactionary solution of 100μl total volume, with regard to 2 μl of 10U/μl DNA polymerase I, 6μl of DNAseI is presented in the 100μl total volume as an activity of from about 10 mU to about 350 mU, preferably from about 25 mU to about 200 mU, and more preferably from about 50 mU to about 150 mU. As far as volume ratio in the essential optimum reaction conditions aforenoted is kept at the certain level, volume of each enzyme and total volume of the reactionary solution can be changed optionally. In the other words, volume of DNAseI may be adjusted, with regard to 20 U of DNA polymerase I in the total volume of 100μl, to from about 10 mU to about 350 mU, preferably from about 25 mU to about 200mU, and more preferably from about 50 mU to about 150 mU. Turning to the other aspect, it is recommended to perform nick translation reaction by adjusting volume of DNAseI, with regard to 1 Unit of DNA polymerase I, to from about 0.5/1,000 U to about 17.5/1,000 U, preferably from about 1.25/1,000 U to about 10/1,000 U, and more preferably from about 2.5/1,000 U to about 7.5/1,000 U. Then, with regard to 1 μg of DNA, volume of DNA polymerase I is adjusted to about 10 U, while that of DNAseI is adjusted to from about 5 mU to about 175mU, preferably from about 12.5 mU to about 100 mU, and more preferably from

aout 25 mU to about 75mU. With regard to the other probes, an amount of DNA as well as optimum reaction conditions on DNA polymerase and DNAse I are determined by referring to the optimum reaction conditions aforenoted, then the probe length (the length of from about 350 bases to about 600 bases) is also determined in which the length may allow the probes to be effectively labelled and may allow the probes to be effectively *in situ* hybridized to the exogenous microorganisms DNA.

Stringent condition to be employed at the *in situ* hybridization is a condition, for example, to incubate under the presence of formamide of from about 30% to about 60%, preferably at about 50%, at from about 30 ℃ to about 50 ℃, preferably from about 38℃ to about 42℃, and rinse successively.

After *in situ* hybridization, blocking operation may also be performed. In particular, 1 ml of Blocking reagent (prepared with sterilized-purified water to be 10ml as their total volume containing 2 ml of Rabbit Normal Serum and 0.5ml of PBS Raw Solution) per single slide is dropped onto the smeared area thereof and the slides were left for from 15 to 60 minutes. Then, Blocking reagent is removed.

In order to detect the signals resulted from hybridization with the bacterial gene (genome DNA or RNA), any coloration reaction utilizing the conventional antigen-antibody reaction or the like may be employed. Namely, the samples so hybridized are fuly washed, then are subjected to Blocking, and are treated with complexes like anti-FITC antibody, anti-digoxigenin antibody, for example, alkaline phosphatase complexes, followed by evaluation on the hybridization results through signals to be expressed by the chromogenic substrates in the complexes. For example, when a probe is labelled with digoxigenin 11-dUTP as noted above, anti-digoxigenin alkaline phosphatase complexes will be employed, and the probe may be detected with a substrate (Nitro Blue Tetrazolium, 5-Bromo-4-Chloro-3-Indolyl Phosphate or the like) to be usually employed on the alkaline phosphatase.

Then, the smear samples, which are washed after the coloration reaction, are subjected to counterstain with Naphtol Black, Fast Green (20mg/50ml, Wako Chemicals) or the like, and in-cell signals are detected by an optical microscopy.

Particularly, in order to take signals in hybridization by employing, for example, DNA probes labelled with digoxigenin as detective DNA probes, labelled antibody solution is prepared by diluting from 10 to 200-fold, preferably 50-fold the labelled antibody (prepared with 12.6 μl of Buffer A (prepared with quantum sufficient of sterilized-purified water to be 100 ml as their total volume containing 746 mg of Triethanolamine, 17.5 mg of Sodium Chloride, 20.3 mg of Magnesium Chloride Hexahydrate, 1.36 mg of Zinc Chloride, 1000 mg of Bovine Serum Albumin and quantum sufficient of Hydrochloric Acid) to be 14 μl as their total volume containing 1.05 Unit of alkaline-phosphatase-labelled anti-digoxigenin antibody solution) with the antibody dilution (prepared with quantum sufficient of sterilized-purified water to be 0.7ml as their total volume containing 8.48 mg of Tris-(Hydroxymethyl)-Aminomethane, 6.14 mg of Sodium Chloride, and quantum sufficient of Hydrochloric Acid), then 10 μl of the labelled antibody solution is dropped onto each of the smeared area, and they may be left for from 15 to 60 minutes. After then, they are immersed in from two-fold to fifty-fold diluted solution, preferably ten-fold diluted solution of the labelled antibody detergent solution (prepared with sterilized-purified water to be 100ml as their total volume containing 1ml of Polysolvate 20 and 50ml of PBS Raw Solution) and are centrifuged for from five to 30 minutes during which they are being immersed in the detergent solution. This operation is repeated twice, then the samples are immersed in the treatment solution prepared by mixing Preliminary Treatment Solution 1 (prepared with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 6.06 g of Tris-(Hydroxymethyl)-Aminomethane, 2.92 g of Sodium Chloride, and quantum sufficient of Hydrochloric Acid) with equal amount of Preliminary Treatment Solution 2 (prepared with quantum

sufficient of sterilized-purified water to be 50ml as their total volume containing 5.08g of Magnesium Chloride Hexahydrate), then diluting approximately 5-fold with sterilized-purified water, and are centrifuged for from five to 30 minutes during which they are being immersed in the detergent solution. 1 ml of chromogenic agent (Nitro Blue Tetrazolium (NBT)/ 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) per single slide is dropped onto the smeared area of the slides by filtrating with a disposable syringe equipped with 0.2μm syringe top filter, and is kept in the dark and is left in a wet chamber for from about 15 minutes to about 60 minutes at from about 10℃ to about 45℃, preferably at 37℃. Then, they are immersed for from about two to about 10 minutes in the solution prepared by diluting from two-fold to about 50-fold, preferably about ten-fold the chromogenic agent cleaner (prepared with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 606mg of Tris-(Hydroxymethyl)-Aminomethane, quantum sufficient of Hydrochloric Acid and 186mg of Disodium Ethylenediaminetetraacetic acid Dihydrate) and are air-dried. Further, they are immersed in the solution prepared by diluting from two-fold to 50-fold, preferably 10-fold the counterstain solution (prepared with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 50mg of Fast Green FCF (Edible Dye Green No.3)) and in an acetic acid solution of from about 0.1% to about 5%, preferably about 1%. Thereafter, they may be immersed again in the solution prepared by diluting about two-fold to about 50-fold, preferably 10-fold the cleaner aforenoted to remove excessive counterstain solution and are completely air-dried. Each of such chromogenic agents may be prepared individually.

Preferable solution of the anti-digoxigenin antibodies labelled with alkaline phosphatase may include a solution which will offer color in the blotted area made by blotting one ng of DNA labelled with digoxigenin onto a blotting membrane, blocking the same, then treating those with the 10,000-fold diluted solution of the anti-digoxigenin antibody labelled with

alkaline phosphatase, and reacting those with chromogenic substrates (NBT/BCIP), while it will not offer any color according to the same procedure employing DNA without digoxigenin label. Anti-digoxigenin antibodies taken from sheep are preferable. In particular, it is recommended to take such antibodies by purifying the immunized sheep serum with an ion-excgange chromatography and an antibody column chromatography.

Chromogenic agent (NBT/BCIP solution, pH 9.0-10.0) is an agent prepared preferably with quantum sufficient of sterilized-purified water to be 10ml as their total volume containing 3.3mg of Nitro Blue Tetrazolium (NBT), 1.65mg of 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP), 99μg of N,N-dimethyl-formamide, 121mg of Tris-(Hydroxymethyl)-Aminomethane, quantum sufficient of Hydrochloric Acid, 58.4mg of Sodium Chloride, 101.6mg of Magnesium Chloride Hexahydrate.

Preferable chromogenic agents may include an agent which will offer violet signals in the blotted area made by blotting proteins labelled with alkaline phosphatase onto a blotting membrane and treating the same with such chromogenic agents in the dark at room temperature.

In the counterstain above, edible dye, for example, Yellow No.4 (Tartrazine) can be used to present more clearly contrast between signals and cells. Factors of such poor countercontrast may include similarity of the colors to be expressed, namely, between violet color offered by the substrate and blue color offered by Naphtol Black. When this methodolofy is applied to the present invention, it came to know that such methodology is useful at the counterstain. Any conventional methodology has never employed a food dye.

Nick translation methodology is applicable as a method to label the digoxigenin. The other methodology may include, for example, PCR Method, Random Primer Labelling Method, *in vitro*

Transcription Labelling Method, Terminal Transferase Labelling Method or the like.

When at least one expressed violet signal(s) is/are confirmed by the optical microscopy (×1,000) on the subjected cells in the single well which is stained with the counterstain solution aforenoted, the sample may be designated as positive.

Then, Japanese Patent Nos. 2558420, 2798499, 2965543, 2965544 and 3026789 can be referred at the producing of the detective probes.

For example, in order to culture bacteria taken from working-cell-banks, the working-cell-banks (SA-24) are smeared streakily with a platinum loop, a disposable plastic loop or the like onto L-broth solid medium containing 50 μg/ml ampicillin mounted on the sterilized laboratory dishes (Screening).

After an overnight cultivation thereof, single colony so cultured is inoculated into 5 ml of L-broth medium containing 50μg/ml ampicillin and is shaking cultured overnight at 37℃ (Precultivation).

2.5 ml of the culture solution is inoculated individually into 400ml of the medium in a flask and is shaking cultured overnight at about 37℃ (Main Cultivation).

Then, in order to extract SA-24 plasmid DNA, the cultured solution prepared through the main cultivation are centrifugated at 4℃, in 4,000×g, for 10 minutes to collect the bacteria. Supernatant are removed, 20ml of STE (10 mmol/l Tris-HCl (pH 8.0), 1 mmol/l Disodium Ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/l Sodium Chloride) are added to the remained bacteria to resupend the same, and they are centrifugated at 4℃, in 4,000 ×g, for 10 minutes to collect the bacteria. The bacteria is suspended with 5 ml of Solution-1 (50 mmol/l glucose, 25 mmol/l

Tris-HCl (pH 8.0), 10 mmol/l EDTA) containing 10mg/ml Lysozyme and is left for five minutes under the room temperature. Thereafter, 10 ml of Solution-2 (0.2 mmol/l Sodium Hydroxide, 1% Sodium Dodecyl Sulfate (SDS)) is added, then they are upset to mix, and are left for ten minutes on ice. 7.5 ml of Iced Solution-3 (3 mol/l potassium acetate (pH 4.8)) is added, then they are upset to mix, and are left for ten minutes on ice.

After centrifugation by a high speed refrigerated centrifuge at 4° C for 30 minutes at 45,000×g, the supernatant is recovered, and left to stand to cool to room temperature. After leaving to stand, 0.6 volume of isopropanol (about 24 ml) is added thereto, mixed by inversion and left to stand at room temperature for 5 minutes or longer. After centrifugation by a high speed refrigerated centrifuge at 25° C, for 30 minutes at 28,000×g, the supernatant is discarded, and thus resulting pellet is washed with 70% ethanol and air dried. After air drying, 8 ml of TE (10 mmol/l Tris-hydrochloric acid (pH 8.0), 1 mmol/l EDTA) is added thereto to dissolve the pellet (extraction of plasmid DNA).

Next, for the purification of the plasmid DNA containing SA-24, 800μl of 10 mg/ml ethidium bromide and 8.6 g of cesium chloride are added to the resulting plasmid DNA followed by mixing by inversion to dissolve the plasmid. The solution is placed in a centrifuge tube, which is then capped or sealed. After centrifugation at 20℃ for 5 hours at 500,000×g with a vertical rotor, a band of the plasmid DNA is fractionated using a glass syringe or an injection needle under the irradiation of an ultraviolet ray light. To the fractionated plasmid DNA solution is added an equivalent amount of TE-saturated 1-butanol followed by mixing by inversion and centrifugation at 15,000 ×g for 5 minutes by a high speed microcentrifuge to remove the supernatant. This operation is repeated to eliminate ethidium bromide in the plasmid DNA solution. Next, thereto is added TE to give the volume of 1.5 ml followed by desalting on a demineralization column (NAP-10). To the desalted plasmid DNA

solution is added 30μl of a 3 mol/l sodium acetate solution followed by mixing, and 3 fold amount of 99.5% ethanol is added thereto followed by mixing by inversion and leaving to stand at -20℃ for 30 minutes or longer. After leaving to stand, centrifugation is conducted with a high speed refrigerated micro centrifuge at 4℃ for 20 minutes at 15,000×g to remove the supernatant. Thereafter, cold 70% ethanol is added thereto to suspend therein, and once again, centrifugation is conducted with a high speed refrigerated micro centrifuge at 4℃ for 20 minutes at 15,000×g to remove the supernatant. Thus resulting precipitate of the plasmid DNA is evaporated to dryness under a reduced pressure. TE in an amount of 100μl is added to the plasmid DNA to dissolve completely, and the concentration is measured on the basis of the absorbance at 260 nm (Purification of plasmid DNA containing SA-24). Then, size check of the plasmid DNA containing SA-24 is carried out by a treatment with arestriction enzyme and agarose electrophoresis.

In order to purify SA-24 by digesting the plasmid DNA containing SA-24 with restriction enzyme(s) and successively applying them to an agarose electrophoresis, 1 mg of the plasmid DNA with SA-24, molecular weight of which had been determined, are digested through a reaction to be performed at 37℃ for one and half hours or more under the presence of the restriction enzyme HindIII alone or the combination of HindIII and the other restriction enzyme(s). After digesting the plasmid DNA, completion of such digestion is confirmed by applying a part of the reactionary solution to 0.8% agarose electrophoresis. Then, after confirming such digestion, SA-24 band is taken by applying the solution to an electrophoresis with 0.8% agarose as a fractionator. SA-24 so taken are extracted from the agarose gel and are purified, then, concentration thereof is determined with an absorptiometer. A part of the purified SA-24 is applied to an electrophoresis with 0.8% agarose gel and is appeared as a single band.

SA-24 may preferably be labelled with digoxigenin with the

reactionary solution having the composition listed in the following Table 1 including 2 μg of the purified SA-24.

TABLE 1

Composition in Reactionary Solution to Label

	Amount (μL)
DNA probe	X
10×L.B.[a]	10
100mmol/L dithiothreitol	10
dNTPs[b] (A、G、C 0.5mmol/L)	4
digoxigenin-dUTP[c] (0.4mmol/L)	5
DNAseI[d]	6
10U/μL DNA polymerase I	2
Sterilized-Purified Water	Y
Total	100

[REMARKS]

(a) 10×L.B. : 0.5 mol/L Tris-HCl (pH 7.5)、
50 mmol/L magnesium chloride、
0.5 mg/mL bovine serum albumin

(b) dNTPs : 0.5 mmol/L 2'-dioxyadenosine-5'-triphosphate、
0.5 mmol/L 2'-dioxyguanosine-5'-triphosphate、
0.5 mmol/L 2'-dioxycytidine-5'-triphosphate

(c) digoxigenin-dUTP : 0.4mmol/L digoxigenin-11-2'-dioxy-uridine-5'-triphosphate

(d) DNAse I : Deoxyribonuclease I is diluted with 25 mmol/L Tris-HCl (pH 7.5), 50% glycerin solution to be used at 50-150 mU per total amount of 100μL and is adjusted to the amount aforenoted.

In Table 1, the volume **X** is adjustable to realize preferable probe concentration aforenoted according to the concentration of raw probe solution, then, the final volume is adjusted by determining, based on the volume **X**, the volume **Y** of Sterilized- Purified Water. After putting the labels, the

reaction is terminated by adding 100μl of TE to the reaction solution.
Free nucleotides are then removed by adding solution to terminate the reaction into the spin column and centrifuging the same at 4 ℃, in 380×g, for 10 minutes. Thereafter, concentration of the eluted solution is determined with an absorptiometer and is adjusted with TE to the unit level of ng/μl.

To confirm the labelled subjects, 0.5μl of the labelled SA-24 is dropped onto a membrane and is air-dried. The membrane is immersed into the blocking reagent and is kept for 30 minutes under the room temperature. The membrane is then immersed for 30 minutes under the room temperature into the solution of anti-digoxigenin antibody labelled with alkaline phosphatase prepared by diluting 5,000-fold with a solution containing 0.1mol/l Tris-HCl (pH 7.5) and 0.15 mol/l sodium chloride. The membrane is immersed into a solution containing 0.1 mol/l Tris-HCl (pH 7.5) and 0.15 mol/l sodium chloride, then is shaked for 10 minutes under the room temperature, and is rinsed twice. Further, the membrane is immersed for 10 minutes under the room temperature into a solution containing 0.5 mol/l Tris-HCl (pH 9.5), 0.15 mol/l sodium chloride and 50 mmol/l magnesium chloride. The membrane is then immersed into chromogenic agent for 10 minutes, in the dark, under the room temperature. Labelled subjects are confirmed with violet color appeared under the spotted position.

Spin column is produced by filling a few amount of sterilized grass wool into 1 ml volume of disposable syringe. Sephadex G-50 swelled with 1 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA and 0.1% SDS is then filled into the syringe. The syringe is inserted into 15ml volume of the disposable conical tube and is centrifuged at 4℃, in 320×g, for 10 minutes to remove the excess buffer solution. The syringe is then detached from the disposable conical tube and the excess buffer solution is discarded. Thereafter, the spin column is assembled by engaging 1.5ml volume of Eppendolf Tube with the bottom of the disposable

conical tube and entering the syringe into the conical tube.

Dot Blot Hybridization according to the following procedure is recommended to confirm specificity of the probes.

First of all, in order to denature each of the spotted genomic DNA, according to the standard procedure, 100ng of the various bacterial genome so prepared are spotted onto nylon membranes (Pall BioDine Type B; Nihon Pall Ltd.), and air-dried membranes are kept for 10 minutes on the filter papers (3mm: Wattman) saturated with solution containing 0.5mol/l sodium hydroxide and 1.5mol/l sodium chloride. The denatured DNA are then neutralized by leaving them for 10 minutes on the previously noted filter papers saturated with solution containing 0.5mol/l Tris-HCl (pH 7.5) and 1.5 mol/l sodium chloride. They are further left for five minutes on the previously noted filter papers saturarted with 2 × SSC (Standard Saline Citrate) Solution and are rinsed. After then, such membranes are air-dried and are immersed into 2 ×SSC Solution for five minutes. In accordance with the standard procedure, the membrances are immersed into Prehybridization Solution in plastic bags and are kept at 42℃ for 60 minutes. In the plastic bag, the membranes are immersed in 15 ml of Hybridization Solution containing 400ng of Probes and are reacted overnight at 42℃. Next, the membranes are immersed into the solution containing 2 ×SSC and 0.1% SDS (sodium dodecyl sulfate) and are rinced for five minutes (this is repeated twice). The membranes are then immersed into the solution containing 0.1×SSC, 0.1% SDS and are rinced for ten minutes (this is repeated three times). The membranes are immersed into 2×SSC solution and are rinced for five minutes. The membranes are immersed into the solution containing 3% bovine serum albumin, 1% Blocking Buffer (Boehringer), 0.1 mol/l Tris-Hcl (pH 7.5) and 0.15mol/l Sodium Chloride, and are gently shaked for 30 minutes. The membranes are immersed into the solution prepared by diluting 5,000-fold the alkaline-phosphatase labelled anti-digoxigenin antibody (Boehringer) with the solution containing 0.1 mol/l Tris-HCl (pH 7.5) and 0.15 mol/l Sodium Chloride, and are gently shaked for 30 minutes.

Next, the membranes are immersed into the solution containing 0.1 mol/l Tris-HCl (pH 7.5) and 0.15 mol/l sodium chloride, and are sahked for 15 minutes (twice). The membranes are immersed into the solution containing 0.1 mol/l Tris-HCl (pH 9.5), 0.1 mol/l Sodium Chloride and 5 mmol/l Magnesium Chloride, and are sahked for 5 minutes. The membranes are immersed into NBT-BCIP Solution (GIBCO BRL) and are subjected to chromogenic reaction in the dark. The membranes are then immersed into TE (10 mmol/l Tris-HCl (pH 8.0), 1 mmol/l EDTA) to terminate the chromogenic reactionand are air-dried. Particulars of Prehybridization Solution and Hybridization Solution are illustrated in the following Table 2.

TABLE 2

[Unit : ml]

	Prehybridization Solution	Hybridization Solution
Formamide	7. 5	6. 7 5
20×SSC Solution	3. 7 5	3. 7 5
100×Denhart Solution	0. 7 5	0. 1 5
0.5mol/L Phosphatized Buffer Solution	0. 7 5	0. 6
Sterized-Distillated Water	1. 5	1. 9 5
10mg/mL Salmon Sperm DNA	0. 7 5	0. 3
50% Dextran Sulfate	-----	1. 5
Total Liquid Amount	1 5. 0	1 5. 0

Conventional surfactants can be employed as those to be used in the step of *in situ* hybridization. Typically, the surfactants are roughly divided into anionic surfactant, non-ionic surfactant, cationic surfactant and amphoteric surfactant.

Anion surfactants are also referred to as anionic surfactants, which yield an organic anion upon ionization in water. When a lipophilic group in the molecule of the surfactant is represented by R, examples of the anion surfactant include RCOONa, RSO_3Na, RSO_4Na and the like. An aqueous solution of the surfactant containing a weakly acidic group such as RCOONa is liable to be hydrolyzed and is weak alkaline. However, an aqueous solution of a surfactant having a strongly acidic group such as RSO_3Na, RSO_4Na or the like is resistant to hydrolysis, which shall be neutral. Because it is anionic, it may lose surface activity in the presence of a large quantity of cationic substance, and may be inactivated in a strongly acidic circumstance.

Nonionic surfactants refer to those having a hydrophilic group which is nonionic. An ethylene oxide group ($-CH_2CH_2O-$) is often used as the hydrophilic group. As number of this group increases, hydrophilicity is increased. To the contrary, as number of the lipophilic group increases, lipophilicity is increased. Therefore, it is characterized in that surfactants with variously altered hydrophilicity and lipophilicity can be obtained. Because a nonionic surfactant does not ionize in water and is hardly affected by inorganic salts, less action is exerted also on a living body. In addition, the detergent action thereof is potent with comparatively less foaming, therefore, it is widely used not alone as a detergent, but in pharmaceuticals, cosmetics, foods and the like. Water soluble nonionic surfactant becomes insoluble in water at a certain temperature as the temperature rises, and then the aqueous solution starts to be turbid. Such turbidity results from the cleavage of hydrogen bonds between the hydrophilic groups and water.

Cation surfactants are also referred to as cationic surfactants, which yield an organic cation upon ionization in water. Although cation surfactants do not have potent detergent action in general, they strongly bind to anionic substances such

as bacteria, leading to a great bactericidal action. Moreover, they also have an anti-static ability for fibers and plastics. Although dodecyltrimethyl chloride $[C_{12}H_{25}(CH_3)_3N]Cl$ as a typical exemplary cation surfactant is water soluble, didodecyldimethylammonium chloride $[(C_{12}H_{25})_2(CH_3)_2N]Cl$ is insoluble in water, which forms a vesicle in the form of a bimolecular film in water, and is soluble in benzene.

Ampholytic surfactants are surfactants having both an anionic group and a cationic group in the molecule. Ionization state thereof in water is similar to those of amino acids, and thus many of ampholytic surfactants are amino acid derivatives. Therefore, they have an isoelectric point similarly to amino acids, which act as an anion surfactant in an alkaline region from the isoelectric point, whilst as a cation surfactant in an acidic region. Water solubility becomes the lowest at the isoelectric point, and the surface tension is also reduced. Ampholytic surfactants are used for a bactericide, an antistatic agent or the like.

Furthermore, anion surfactants are classified into the carboxylic acid type, sulfonic acid type, sulfate ester type and phosphate ester type, whilst nonionic surfactants are classified into the ester type, ether type, ester ether type and alkanolamide type. Cation surfactants are classified into alkylamine salt type and quaternary ammonium salt type, whilst ampholytic surfactants are classified into carboxy betaine type, 2-alkylimidazoline derivative type and glycine type.

Moreover, the anion surfactants of carboxylic acid type are further classified into fatty acid monocarboxylate salts, N-acylsarcosine salts and N-acylglutamate salts. Representative examples thereof respectively include: sodium laurate and medicated soap as the fatty acid monocarboxylate salts; sodium N-lauroylsarcosine as the N-acylsarcosine salt; and disodium N-lauroylglutamate as the N-acylglutamate. Still more, the sulfonic acid type is further classified into dialkyl

sulfosuccinate salts, alkane sulfonate salts, alpha-olefin sulfonate salts, straight chain alkyl benzenesulfonate salts, alkyl (branched chain) benzenesulfonate salts, alkyl naphthalenesulfonate salts, naphthalenesulfonate salts-formaldehyde condensates and N-methyl-N-acyltaurine salts. Representative examples include: sodium dioctyl sulfosuccinate as the dialkyl sulfosuccinate salt; sodium dodecane sulfonate as the alkane sulfonate; sodium straight chain dodecyl benzenesulfonate as the straight chain alkyl benzenesulfonate salt; sodium dodecyl benzenesulfonate as the alkyl (branched chain) benzenesulfonate salt; sodium butyl naphthalenesulfonate as the alkyl naphthalenesulfonate salt; and sodium N-methyl-N-stearoyltaurine as the N-methyl N-acyltaurine salt. In addition, the sulfate ester type is further classified into alkyl sulfate salts, polyoxyethylene alkyl ether sulfate salts and oil-and-fat sulfate ester salts. Representative examples include sodium dodecyl sulfate, sodium lauryl sulfate and sodium cetyl sulfate as the alkyl sulfate salt; and polyoxyethylene lauryl ether sulfate triethanolamine as the polyoxyethylene alkyl ether sulfate salt. Moreover, the phosphate ester type is further classified into alkyl phosphate salts, polyoxyethylene alkyl ether phosphate salts and polyoxyethylene alkylphenyl ether phosphate salts. Representative examples include disodium monolauryl phosphate as the alkyl phosphate salt; and sodium polyoxyethylene lauryl ether phosphate and polyoxyethylene oleyl ether phosphate (8MOL) as the polyoxyethylene alkyl ether phosphate salt.

Ester type of the nonionic surfactants is further classified into fatty acid glycerin, fatty acid sorbitan and fatty acid sucrose ester. Representative examples respectively include: glycerin monostearate as the fatty acid glycerin; sorbitan monostearate, sorbitan trioleate, sorbitan sesquioleate, sorbitan monolaurate, polysorbate 20 (polyoxyethylene sorbitan fatty acid ester), polysorbate 60 and polysorbate 80 as the fatty acid sorbitan; and stearic acid sucrose ester as the fatty acid sucrose ester. Additionally,

the ether type is further classified into polyoxyethylene alkyl ether, polyoxyethylene alkyl phenyl ether and polyoxyethylene polyoxypropylene glycol. Representative examples include: polyoxyethylene lauryl ether, polyoxyethylene stearyl ether and polyoxyethylene cetyl ether as the polyoxyethylene alkyl ether; and polyoxyethylene nonyl phenyl ether and polyoxyethylene octyl phenyl ether as the polyoxyethylene alkyl phenyl ether. In addition, the ester ether type is further classified into fatty acid polyethylene glycol and fatty acid polyoxyethylene sorbitan. Representative examples thereof respectively include oleic acid polethylene glycol as the fatty acid polyethylene glycol; and polyoxyethylene sorbitan palmitate and polyoxyethylene sorbitan monolaurate as the fatty acid polyoxyethylene sorbitan. In addition, the alkanolamide type involves only fatty acid alkanolamide alone. Representative example is lauric diethanolamide.

The alkyl amine salt type of the cation surfactant includes monoalkyl amine salts, dialkyl amine salt and trialkyl amine salts. Representative examples thereof include monostearyl amine hydrochloride. Moreover, the quaternary ammonium salt type is further classified into alkyltrimethyl ammonium chloride (or bromide or iodide), dialkyldimethyl ammonium chloride (or bromide or iodide), and alkyl benzalkonium chloride. Representative examples respectively include: stearyltrimethyl ammonium chloride as the alkyltrimethyl ammonium chloride (or bromide or iodide); distearyldimethyl ammonium chloride as the dialkyldimethyl ammonium chloride (or bromide or iodide); and lauryl benzalkonium chloride as the alkyl benzalkonium chloride.

The carboxy betaine type of the ampholytic surfactant is only alkyl betaine alone. Representative example is lauryl betaine. Additionally, the 2-alkyl imidazoline derivative type is only 2-alkyl-N-carboxymethyl-N-hydroxyethyl imidazolinium betaine alone. Representative example includes 2-undecyl-N-carboxymethyl-N-hydroxyethyl imidazolinium betaine. In addition, the glycine type may be alkyl (or dialkyl) diethylene

triaminoacetic acid, and the representative example includes dioctyl diethylene triaminoacetic acid.

Moreover, in addition to the representative examples as described above, Triton X-100, lauryl sarcosine, saponin, BRIJ35, alkyl allyl polyether alcohol, higher alcohol sulfate, N-cocoyl-L-arginine ethyl ester DL-pyrrolidone carboxylate salt, sodium N-cocoyl-N-methyl aminoethyl sulfonate, cholesterol, self emulsifying type monostearate glycerin, squalane, stearyl alcohol, stearic acid polyoxyl 40, cetyl alcohol, cetomacrogol 1000, sebacate diethyl, nonylphenoxy polyoxyethylene ethane sulfate ester ammonium, polyoxyethylene oleylamine, polyoxyethylene sorbit yellow bees wax, polyoxyl 35 castor oil, macrogol 400, N-coconut oil fatty acid acyl L-arginine ethyl·DL-pyrrolidone carboxylate salt, lauryldimethylamine oxide solution, lauromacrogol, methylcellulose, CMC (carboxymethylcellulose), polyoxyethylene hardened castor oil 20 and polyoxyethylene hardened castor oil 60, CHAPS, deoxycholic acid, digitonin, n-dodecyl maltoside, Nonidet P40, n-octyl glucoside, octyl thioglucoside, laurate sucrose, dodecyl poly(ethylene glycol ether)n,n-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate and the like are also included.

It is important to apply the various surfactants listed above at the *in situ* hybridization step, while application embodiment thereof is not limited anyway. For example, one skilled in the art would mix the surfactants previously into probe solution or probe dilution, otherwise, the skilled artisan may prepare the separate soulution containing surfactants and add the same before, simultaneously or after application of the probe solution onto the area to be smeared.

If any positive control probe is necessary in the present invention, such probe may be prepared as follows. For example, in order to conduct the extraction and purification of the genomic DNA of U937 cell (ATCC CRL-1593.2), U937 cells are first

cultured in a 5% carbon dioxide gas incubator at 37° C using an RPMI1640 medium (25 ml) in a cell culture flask (175 cm^2). The U937 culture solution is placed in a 50 ml centrifuge tube, and centrifuged at 4° C for 10 minutes at 220×g to recover the U937 cells. The cells are suspended and washed in 10 ml of PBS, and again centrifuged at 4° C for 10 minutes at 180×g to recover the cells. Thereafter, the supernatant is discarded, and the cells are suspended in 1 ml of a TE solution containing 200 µg/ml proteinase K and containing 1% SDS, followed by leaving to stand at 37° C for 30 minutes. Phenol extraction is repeated three to four times to execute deproteinization. Genome deposited through the ethanol precipitation is recovered, dissolved in 500 µl of sterile purified water containing 2.5 µg of ribonuclease, and left to stand at 42° C for 30 minutes. The phenol extraction is repeated two to three times to execute deproteinization. Genome deposited through the ethanol precipitation is recovered, and dissolved in 500 µl of TE. Thereafter, a positive control probe can be produced by measuring the concentration with an absorbance meter, and subjecting to digoxigenin labelling. Moreover, the positive control probe which may preferably used is one which permits to ascertain the hybrid formation when the positive control probe is subjected to dot hybridization on a membrane with 100 ng of U937 genome spotted thereon. When a negative control probe is required, it can be produced by any known method.

Further, the present invention also includes a kit for detecting and/or identifying causative microorganisms of infectious diseases by taking phagocytes from the clinical specimens containing active phagocytes, immobilizing the phagocytes so taken, treating the phagocytes to improve cell membrane permeabilities thereof, further treating the phagocytes to bare DNA in the causative microorganisms which might be existed in the phagocytes, *in situ* hybridizing DNA so bared with detective DNA probe(s) which can hybridize with such bared DNA under stringent conditions, and detecting and/or identifying the causative microorganisms based on signals so

detected, the kit comprises at least one or more enzyme selected from the group consisting of lysostafin, lysozyme, N-acetylmuramidase and zymolase used in the exposing step of the DNA, probe solution containing surfactant, and one or more DNA probe for detection. The kit includes, reagent for separating blood, enzyme pretreatment reagent, enzyme reagent, acetylation reagent, probe solution, blocking reagent, labelled antibody, labelled antibody diluent, coloring pretreatment liquid-1, coloring pretreatment liquid-2, coloring reagent, counter staining solution, PBS stock solution, hybridization stock solution, labelled antibody washing solution, coloring reagent washing solution, APS coated slide glass, probe dilution solution, buffer A and the like as demonstrated in the following Examples. Among these, it is preferred that at least the enzyme reagent and the probe solution are included. In addition, various reagents used in the present invention may be included for example, chloroform, ethanol, acetic anhydride, DMSO, PMSF, formamide, acetic acid, hydrochloric acid, sodium hydroxide and the like. Moreover, instrument and machine such as low speed centrifuge, incubator, counting chamber, shaker, humid box, incubator, light microscope, variable pipette, blood collection tube, tip, pipette, staining bottle, measuring cylinder, glass syringe, 0.2 μm syringe top filter may be included.

Furthermore, the present invention provides a process for monitoring the gene of a foreign microorganism digested by a phagocyte included in a clinical specimen which contains a phagocyte derived from a living body. Moreover, the present invention provides a process for identifying the gene of a microorganism which becomes a candidate of the causative microorganism which a causative microorganism of sepsis or a causative microorganism of bacteremia is specified on the basis of the results identified.

It was revealed that when this process was applied in practice to diagnoses for blood of a variety of patients suspected as suffering from sepsis, causative microorganism

could be detected with about four times higher sensitivity compared to the blood culture process with no influence of the administered antimicrobial agent, and the identity of the detected microorganism strain was favorable. Furthermore, in comparison with the blood culture which requires three days or longer and approximately 14 days for the examination, an accurate result can be achieved by a simple operation within a short time period, i.e., about 8 hours, until the completion of the entire operation, according to the process of the present invention, an useful marker can therefore be realized in monitoring and the like in prognosis or diagnosis of an infectious disease such as sepsis, bacteremia or the like in which a rapid and favorable care is particularly necessary.

EXAMPLES

The present invention will be described in detail along with the following Examples, but as a matter of course would not be limited to the Examples because of the following disclosures.

EXAMPLE 1

Blooding and Preparation of Blood-Specimens

Twelve blood specimens (Specimen A-L) were obtained as a clinical specimen from the patients who are under the suspicion about sepsis. 10ml of heparinized venous bloods were obtained from each patient and were mixed with the blood components separative reagent (adjusted with sterilized-purified water to be 25ml as their final volume containing 225mg of Sodium Chloride and 1.5g of Dextran (Molecular Weight: 200,000-300,000)) in the ratio of 4:1. Leukocyte fractions (the upper layer) were then obtained by leaving them at 37℃ for 30 minutes. Leukocytes were appeared by centrifuging the leukocyte fractions in 160×g, at 4 ℃ and for 10 minutes. Pelletized leukocytes so obtained were suspended with 1 ml of sterilized-purified water and the suspension were immdeditely put into an isotonic state by adding thereto excessive amounts of PBS (prepared by diluting twenty-fold with sterilized-purified water the PBS Raw Solution

which have been adjusted with sterilized-purified water to be 120ml as their final volume containing 18.24 g of Sodium Chloride, 6.012 g of Sodium Monohydrogen Phosphate Didecahydrate and 1.123 g of Sodium Dihydrogen Phosphate Dihydrate). Such suspension were then re centrifuged in 160×g, at 4 ℃ and for 10 minutes.

EXAMPLE 2

Fixation of Leukocytes

APS coated slides were employed wherein they were made by coating 3-aminopropyltriethoxysilane (APS, SIGMA) onto the slides (JAPAN AR BROWN CO., LTD., PRODUCTS ID. S311BL). Namely, such APS coated slides were produced by putting the slides (PRODUCTS ID. S311BL) into holders, immersing them into a diluted neutral detergent to wash the same, removing the detergent well therefrom with tap water then with purified water, leaving them under the higher temperature (100℃ or more) to dry the slides, and leaving the same under the room temperature to cool the slides. After then, these slides were immersed in Acetone containing 2% APS for one minute and were immediately rinsed gently with Acetone then with sterilized-purified water. These slides were then air-dried. Such performance was repeated and includes the immersion of these slides in Acetone containing 2% APS for one minute, immediate gentle rinse of the slides with Acetone and sterilized-purified water, and air-dry of the same. Finally, the slides were dried under the temperature of 42℃ to realize the APS coated slides.

Pelletized leukocytes were prepared by centrifuging leukocyte fractions in 160×g, at 4 ℃ and for 10 minutes. Then, leukocyte population was determined by adding small amount of PBS to the pelletized leukocytes, suspending the same, and counting the population with a hemacytometer. Leukocytes were duly mounted on the APS coated slides by smearing 5μl of the leukocyte suspension into each well of the slides adjusted with PBS to be cell population of 1×10^5cells/well, realizing the extended mono-layer of the leukocytes, and completely air-drying

the same. After then, they were immersed in Carnoy s fixative solution (prepared by mixing Ethanol, Chloroform and Acetic Acid in the volume ratio of 6:3:1) for 20 minutes and were immersed in 75% Ethanol solution for five minutes. Finally, they were completely air-dried.

EXAMPLE 3

Treatment to Improve Permeability of Leukocyte Cell Membrane

They were immersed in PBS for 10 minutes, then in the solution prepared by diluting ten-fold a pretreatment reagent (prepared by mixing 1.25g of Saponin, 1.25 ml t-octylphenoxy-polyethoxyethanol (specific gravity of 1.068-1.075 (20/4℃), pH (5w/v%) 5.5-7.5) and 25ml PBS Raw Solution, and adjusting with sterilized-purified water to be 50ml as their final volume), and were applied to a centrifuge for 10 minutes to accelerate thier permeability.

EXAMPLE 4

Treatment of Bacterial Cell Wall with Lytic Enzymes

In order to bare DNA in the causative microorganisms, an enzyme solution was prepared by adding, per single slide, 1 ml of a reagent solvent (prepared by diluting 100-fold, with PBS, Dimethyl Sulfoxide (DMSO) containing 0.1 mol/l Phenylmethyl Sulfonylfluoride (PMSF)) to an enzyme reagent (N-acetyl-muramidase 1,000 Units/ml、Lysozyme 100,000 Units/ml and/or Lysostaphin 100 Units/ml), then 1 ml of which were dropped under the temperature of 37℃-42℃ in the wet chamber onto the area where the leukocytes were smeared and were left for 30 minutes. Then, they were immersed in PBS containing 0.2 mol/l Hydrochloric Acid (prepared by adding hydrochloric acid to PBS Raw Solution, diluting 20-fold the same with sterilized-purified water and adujusting final concentration of Hydrochloric Acid to 0.2 mol/l), and were applied to a centrifuge for 10 minutes to accelerate thier permeability.

EXAMPLE 5

Acetylation of Cell Membrane Proteins

Slides were then immersed in the acetylation reagent prepared by adding Acetic Anhydrate to the raw acetylation reagent (adjusted with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 7.46g of Triethanolamine and quantum sufficient of Hydrochloric Acid), diluting 10-fold with sterilized-purified water, and adujusting final concentration of Acetic Anhydride to 0.8%, and were applied to a centrifuge for 10 minutes. After then, they were completely air-dried by immersing them succcecively into 75%, 85% and 98% Ethanol for three minutes respectively.

EXAMPLE 6

Alkalization of Bacterial DNA

(Denaturation of Double-Starnded DNA into Single-Stranded DNA)

Slides were then immersed for three minutes in PBS containing 70mmol/l Sodium Hydroxide (prepared by adding Sodium Hydroxide to PBS Raw Solution, diluting 20-fold with sterilized-purified water, and adujusting final concentration of Sodium Hydroxide to 70 mmol/l). After then, they were completely air-dried by immersing them succcecively into 75%, 85% and 98% Ethanol for three minutes respectively.

EXAMPLE 7

Hybridization

Probe Solution (1.0 ng/μl) containing 15ng of DNA probes labelled with digoxigenin prepared with the diluted probe solution (containing 0.25% SDS, 600μl of Salmon Sperm DNA, 50 μl of 100×Denhurt`s Solution, 500μl of Hybridization Raw Solution, 2250μl of Formamide and 1000μl of 50% Dextran Sulfate) was applied to the smeared area and was left for two hours under the temperature of 37℃-42℃ in the wet chamber. Probe Solution without SDS was employed as a control. DNA probes labelled with digoxigenin were prepared through nick translation methodology. Then, three stained bottles containing the hybridization detergent (prepared by mixing

Hybridization Raw Solution (prepared with sterilized-purified water to be 75ml as their total volume containing 13.15g of Sodium Chloride, 6.615g of Trisodium Citrate Dihydrate) in the ratio of Hybridization Raw Solution : sterilized-purified water : Formamide = 5:45:50) were provided and they were successively immersed therein at 42 ℃ for 10 minutes respectively.

After then, they were centrifuged for 10 minutes during which they were being immersed in PBS. As DNA probes labelled with digoxigenin, probes from *Staphylococcus aureus* of SA-24 (SEQ ID NO: 1), SA-36 (SEQ ID NO: 2) and SA-77 (SEQ ID NO: 3) as well as those from *Staphylococcus epidermidis* of SE-22 (SEQ ID NO: 4), SE-3 (SEQ ID NO: 5) and SE-32 (SEQ ID NO: 6) (See, Japanese Patent No.2798499) were employed. Also, as a probe from *Pseudomonas aeruginosa*, thet of P2-2 (SEQ ID NO: 7) (See, Japanese Patent No.2965544) was employed. Then, as probes from *Enterococcus faecalis*, those of EF-1 (SEQ ID NO: 8), EF-27 (SEQ ID NO: 9) and EF-7 (SEQ ID NO: 10) (See, Japanese Patent No.2965543) were employed. Then, probes from *Escherichia coli* of EC-24 (SEQ ID NO: 11), EC-34 (SEQ ID NO: 12) and EC-39 (SEQ ID NO: 13), that from *Enterobacter cloacae* of ET-49 (SEQ ID NO: 14), and that from *Klebsiella pneumoniae* of KI-50 (SEQ ID NO: 15) (See, Japanese Patent No. 3026789) were also employed. Further, as probes from *Candida albicans*, those of CA-26 (SEQ ID NO: 16), CA-26-1 (SEQ ID NO: 17), CA-26-2 (SEQ ID NO: 18) and CA-26-3 (SEQ ID NO: 19) (See, Japanese Patent No. 2558420) were employed. The present probes were produced with the probes listed above through nick-translation methodologies.

EXAMPLE 8

Blocking

After *in situ* hybridization, blocking operation was performed. 1 ml of Blocking reagent (prepared with sterilized-purified water to be 10 ml as their final volume containing 2ml of Rabbit Normal Serum and 0.5 ml of PBS Raw Solution) per single slide was dropped onto the smeared area thereof and the slides

were left for 30 minutes. Then, Blocking reagent was removed.

EXAMPLE 9

Reaction with Labelled Antibody

Labelled antibody solution was prepared by diluting 50-fold the labelled antibody (prepared with 12.6 μl of Buffer A (prepared with quantum sufficient of sterilized-purified water to be 100 ml as their total volume containing 746 mg of Triethanolamine, 17.5 mg of Sodium Chloride, 20.3 mg of Magnesium Chloride Hexahydrate, 1.36 mg of Zinc Chloride, 1000 mg of Bovine Serum Albumin and quantum sufficient of Hydrochloric Acid) to be 14 μl as their total volume containing 1.05 Unit of alkaline-phosphatase-labelled anti-digoxigenin antibody solution) with the antibody dilution (prepared with quantum sufficient of sterilized-purified water to be 0.7 ml as their total volume containing 8.48 mg of Tris-(Hydroxymethyl)-Aminomethane, 6.14 mg of Sodium Chloride, and quantum sufficient of Hydrochloric Acid), then 10 μl of the labelled antibody solution were dropped onto each of the smeared area, and they were left for 30 minutes. After then, they were immersed in the ten-fold diluted solution of the labelled antibody detergent solution (prepared with sterilized-purified water to be 100ml as their total volume containing 1ml of Polysolvate 20 and 50ml of PBS Raw Solution) and were centrifuged for 10 minutes during which they were being immersed in the detergent solution. This operation was repeated twice, then the samples were immersed in the treatment solution prepared by mixing Preliminary Treatment Solution 1 (prepared with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 6.06 g of Tris-(Hydroxymethyl)-Aminomethane, 2.92 g of Sodium Chloride, and quantum sufficient of Hydrochloric Acid) with equal amount of Preliminary Treatment Solution 2 (prepared with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 5.08 g of Magnesium Chloride Hexahydrate) and diluting 5-fold with sterilized-purified water. The samples were then centrifuged for 10 minutes during which they were being immersed in the treatment solution.

EXAMPLE 10

Detection

1 ml of chromogenic agent (Nitro Blue Tetrazolium (NBT)/ 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) Solution, pH 9.0-10.0: prepared with quantum sufficient of sterilized-purified water to be 10ml as their total volume containing 3.3mg of NBT、1.65mg of BCIP、99μg of N,N-dimethyl-formamide、121mg of Tris-(Hydroxymethyl)-Aminomethane, quantum sufficient of Hydrochloric Acid, 58.4mg of Sodium Chloride, 101.6mg of Magnesium Chloride Hexahydrate) per single slide was dropped onto the smeared area of the slides by filtrating with a disposable syringe equipped with 0.2μm syringe top filter, and was kept in the dark and was left for 30 minutes at 37℃ in a wet chamber. Then, they were immersed for five minutes in the solution prepared by diluting ten-fold the chromogenic agent cleaner (prepared with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 606mg of Tris-(Hydroxymethyl)-Aminomethane, quantum sufficient of Hydrochloric Acid and 186mg of Disodium Ethylenediaminetetra-acetic acid Dihydrate) and were air-dried. Further, they were immersed in the solution prepared by diluting 10-fold the counterstain solution (prepared with quantum sufficient of sterilized-purified water to be 50ml as their total volume containing 50mg of Fast Green FCF (Food Dye Green No.3)) and in the 1% acetic acid solution. Thereafter, they were immersed again in the solution prepared by diluting 10-fold the cleaner aforenoted to remove excessive counterstain solution and were completely air-dried.

EXAMPLE 11

Criterion

When at least one expressed violet signal(s) is/are confirmed by the optical microscopy (×1,000) on the subjected cells which were stained with the counterstain solution in single well, the sample was designated as positive. As the result of the present method, of twelve specimens, bacteria had been detected in the five specimens of Specimen A-SA (*Staphylococcus*

aureus), Specimen F and G-SE (*Staphylococcus epidermidis*), Specimen J-SE and EF (*Enterococcus faecalis*), and Specimen L-SA and CA (*Candida albicans*). In the meantime, when ths same specimens were subjected to the hemoculture methodologies according to the known procedures, although the identical result was confirmed in Specimen A as a detection of SA, no bacteria had been detected in any of Specimen F, G, J and L. Accordingly, it became apparent that the present method could detect those rapidly with better sensitivity in comparison with the hemoculture methodologies.

With regard to the result on Specimen A-SA, the effect offered by adding SDS to the probe dilution is shown in Figure 1. Obviously from Figure 1, detection sensivity on the signal could be improved remarkably with addition of 0.25% SDS. Turning to the other specimens, signals could similarly be detected clearly by adding SDS thereto. Meanwhile, the probes employed in this Example were those produced by performing nick translation on a combination of the base sequences from SA-24 (SEQ ID NO:1), SA-36 (SEQ ID NO:2) and SA-77 (SEQ ID NO:3).

EXAMPLE 12

Investigation on Optimum Cell Population of Leukocytes to be Smeared and Mounted

Optimum Cell Population of Leukocytes to be smeared onto the well (the circular well of 5mm diameter) of APS coated slides had been investigated. 10ml of heparinized healthy human bloods were collected, then, the leukocytes were obtained therefrom according to the procedures of Example 1. Leukocytes so obtained were suspended in the quantum sufficient of PBS and the leukocyte population per 1ml was determined with a hematimeter. A serial dilution commencing at (a) 1×10^8 cells/ml and followed by (b) 5×10^7 cells/ml, (c) 1×10^7 cells/ml, (d) 5×10^6 cells/ml, (e) 1×10^6 cells/ml, (f) 5×10^5 cells/ml and (g) 1×10^5 cells/ml was prepared and 5μl of each population were smeared onto the slides.

Smeared slides were air-dried and were fixed with Carnoy s

fixative solution (See, Example 2). Immediately thereafter, they were stained with the counterstain solution aforenoted and were subjected to the criterion according to the method of Example 11. As a result thereof, the cell population of 1×10^8 cells/ml was too many to detect and was not appropriate. Then, the cell populations of 5×10^6 cells/ml or less were not appropriate, because little population were observed in the well. Accordingly, phagocyte density (x cells/ml) to be immobilized should be adjusted to the range of about 5×10^6 cells/ml$<$x cells/ml$<$about 1×10^8 cells/ml, preferably, to that of about 1×10^7 cells/ml$\leqq$x cells/ml$\leqq$about 5×10^7 cells/ml. It was concurrently apparent therefrom that leukocyte population (y cells/well (5mm diameter)) to be immobilized in the single well of APS coated slides should be adjusted to the range of about 2.5×10^4 cells/well$<$y cells/well (5mm diameter)$<$about 5×10^5 cells/well, preferably, about 5×10^4 cells/well$\leqq$y cells/well (5mm diameter)$\leqq$about 2.5×10^5 cells/well. Experimental results on the samples (a)-(f) were illustrated in Figure 2 (a)-(f) respectively.

EXAMPLE 13

Investigation of Lytic Enzyme to be Employed

Enzymatic conditions to lyse *Staphylococcus aureus* (ATCC 12600), *Staphylococcus epidermidis* (ATCC 14990), *Pseudomonas aeruginosa* (ATCC 10145), *Enterococcus faecalis* (ATCC 19433) and *Escherichia coli* (ATCC 11775) had been investigated. Lysostaphin (Bur. J. Biochem., 38, pp.293-300, 1973) was used as an lytic enzyme for *Staphylococcus aureus* and *Staphylococcus epidermidis*. As for *Enterococcus faecalis*, N-acetylmuramidase (Archs. Oral Biol., 23, pp.543-549, 1978), Lysozyme (SEIKAGAKU CORPORATION) was used. Then, as for *Pseudomonas aeruginosa* and *Escherichia coli*, PBS containing 70 mmol/l soudium hydroxide was used. All of these bacteria were inoculated into 5ml of BHI (Brain Heart Infusion) liguid medium (DIFCO) and were cultivated under 37 ℃ for eight hours or more. Cultivated solution were collected by centrifuging them in $2,000\times g$, at 4 ℃, for 10 minutes. Samples to be subjected were prepared by suspending

the collected bacteria in PBS.

Lysis activities were determined with a microplate reader by evaluating decreased density at 600nm abosorbance on the subjected sample solution. As a result thereof, *Staphylococcus aureus* and *Staphylococcus epidermidis* were lysed with Lysostaphin. As for *Pseudomonas aeruginosa* and *Escherichia coli*, enzyme treatment was not necessary, because they were lysed with PBS containing 70 mmol/l sodium hydroxide. Then, as for *Enterococcus faecalis*, it was discovered that lysis activities therefor were improved by not relying on N-acetylmuramidase alone but on the combination of N-acetylmuramidase with Lysozyme. But, for example, when *Pseudomonas aeruginosa*, *Escherichia coli* or the like are the digested bacteria with phagocytosis, such enzyme treatments may not be necessary, because their cell walls are solved at the alkaline treatment, thereby, their genes are bared. Each enzyme to be employed in the pretreatment procedure of the present invention for solving the ezogenous microorganism would be valid not only for the previously noted bacteria strain, but also for the other bacterial strains including those belonged to *Staphylococcus* genus, *Streptococcus* genus, *Bacillus* genus and *Micrococcus* genus. Such enzymes can be used as an individual enzyme, but to employ them as the mixed enzymes is quite useful. These results were illustrated in Figure 3, in particular, as Figure 3 (a) *Staphylococcus aureus* and *Staphylococcus epidermidis*, (b) *Pseudomonas aeruginosa* and *Escherichia coli*, and (c) *Enterococcus faecalis*.

EXAMPLE 14

Investigation of Enzyme Solution

(Investigation of Optimum Concentration of DMSO)

Since protease contained in the enzyme reagent degrades a form of leukocyte, enzyme activity of DMSO which is solvent of PMSF to be used to keep the form of leukocyte had been investigated. *Enterococcus faecalis* was inoclulated in 50ml of BHI liquid medium noted previously and was cultivated, at 37℃, for eight hours or more. Bacterial cells/ were collected by centrifuging the cultivation liquid, at 4℃, in 2,000×g,

for 10 minutes, then were suspended in PBS and were subjected to heat-treatment in an autoclave (120℃, 10 minutes). Next, these were centrifuged, at 4℃, in 2,000×g, for 10 minutes, then discarding the supernatant, and the precipitates were suspended in 1 ml of PBS and were lyophilized. The lyophilized samples were suspended in 5 mmol/l Tris-HCl (pH 6.0) containing 0-10% DMSO then 2 mmol/l Magnesium Chloride and were designated as a sample on N-acetylmuramidase. *Micrococcus luteus* (JCM1464)was inoclulated in 5ml of BHI liquid medium (supra) and was cultivated, at 37℃, for eight hours or more. Bacterial cells/ were collected by centrifuging the cultivated bacterial solution, at 4℃, in 2,000×g, for 10 minutes. Then the supernatant was discarded, then, bacterial cells/ were collected by rinsing the bacterial pellets though suspension of them in PBS and recentrifuging the same, at 4℃, in 2,000×g, for 10 minutes. The bacteria so collected were suspended in PBS containing 0-10% DMSO and were designated as a sample on Lysozyme. On the other hand, samples on Lysostaphin were also prepared by culturing and collecting *Staphylococcus epidermidis* substantially along with the procedure for the samples on Lysozyme, and suspending the cultured bacteria with PBS containing 0%～10% DMSO.

Enzymatic activities were evaluated by determining, with a micro plate reader, decrease of absorbance at 600nm on the subjected samples. Correlation between DMSO and the enzymatic activities were experimented in this example under the enzymatic titer of (a) N-acetylmuramidase 300 Units/ml, (b) Lysozyme 10,000 Units/ml and (c) Lysostaphin 50 Units/ml. When each enzymatic activity was evaluated with decrease of the density of bacteria suspension (O.D.=600nm) per predetermined time, there is little correlation between DMSO and N-acetylmuramidase activity, while 5 % or more of DMSO lowered activities of Lysozyme and Lysostaphin. There was no decrease on the enzymatic activities in DMSO concentration of 2% or less. Accordingly, concentration of DMSO to solve PMSF is adjusted to less that 5%, preferably 2% or less, more preferably about 1%. Results

were shown in Figure 4 (a)-(c) and in the following Table 3.

TABLE 3
Correlation between DMSO and Enzymatic Activity
(Density Decrease in Bacteria Suspension)

Amounts of DMSO Added (%)	N-acetylmuramidase O.D./5 minutes	Lysozyme O.D./3 minutes	Lysostaphin O.D./10 minutes
0 (control)	79.3±4.8	0.689±0.028	0.168±0.017
0.1	75.0±3.2	0.678±0.026	0.164±0.009
1	75.8±2.8	0.660±0.026	0.160±0.008
2	75.8±2.5	0.653±0.024	0.145±0.009
5	76.3±4.9	0.566±0.017	0.124±0.006
10	73.8±3.5	0.464±0.016	0.094±0.006

Example 15

Examination on Enzymatic Lysis Solution
(Examination on Optimal Concentration of PMSF)

Because protease included in the enzyme reagent deteriorates the morphology of leukocytes, effects of PMSF (manufactured by PIERCE), which is added for the purpose of retaining the morphology of the leukocytes, on enzymatic activity were examined. PMSF was dissolved in 100 µl of DMSO (manufactured by Wako Pure Chemical Industries, Ltd), and diluted to 10 ml with PBS such that the final concentration of PMSF becomes none (0 mmol/l) to 1 mmol/l. To this solution was added proteinase K (manufactured by Boeringer Mannheim) such that titer of the protease becomes 0.2 unit/ml. Heparinized healthy human blood in an amount of 5 ml was collected, and leukocytes were obtained according to the process described in Example 1. Next, the leukocytes were suspended in an appropriate amount of PBS, and the cell number was measured using a counting chamber. Cell number was adjusted to about 5×10^4 cells/well to about 2.5×10^5 cells/well, and 5 µl therefrom was

smeared on the well of the APS coated slide glass. After air drying, fixation was executed according to the method of Carnoy fixation described in Example 2. Using this sample, tests were performed according to the process described in Examples 3 to 11. As a consequence of performing the tests at the concentration of PMSF of 1 µmol/l to 1 mmol/l, effects were found at the concentration of 10 µmol/l or greater, while deterioration of morphology of the leukocytes was completely suppressed at the concentration of PMSF of 0.1 mmol/l or greater. The results are shown in Fig. 5, for (a): protease 0.2 unit/ml alone, (b): 1 µmol/ml PMSF added, (c): 10 µmol/ml PMSF added, (d): 0.1 mmol/ml PMSF added, and (e): 1 mmol/ml PMSF added, respectively.

Example 16

Examination of Optimal Titer of Lytic Enzyme, Zymolase

Optimal titer of zymolase for exposing DNA was examined through lysis of Candida albicans. *Candida albicans* was inoculated in YPD medium, and cultured over day and night at 30° C. Then, two types of the solutions were prepared: a solution of *Candida albicans* as a substrate suspended in PBS (substrate 1); and a solution prepared by Carnoy s fixation, immersing in 70% ethanol, air drying and suspension in PBS (substrate 2). Upon the reaction, a mixture of zymolase/ PBS: 0.5ml, substrate: 1.5 ml, M/15 phosphate buffer: 2.5 ml and sterile purified water: 0.5 ml, adjusted to give the total volume of 5.0 ml was used.

Thereafter, the reaction was allowed at 37° C for 2 hours, and the OD_{800} was measured. Furthermore, the concentration of zymolase (Zymolyase-100T) for use was 0 mg/ml, 0.01 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 2.5 mg/ml and 5 mg/ml. Consequently, each OD_{800} value when the substrate 1 was used was 0.533, 0.521, 0.553, 0.554, 0.548, 0.417, 0.394, 0.288, 0.163 and 0.113, and each OD_{800} value when the substrate 2 was used was 0.445, 0.411, 0.359, 0.282, 0.232, 0.146, 0.115, 0.096, 0.08 and 0.057. It was proven that effectiveness was brought when both of the substrate 1 and substrate 2 were in the range of 0.5 mg/ml to 5 mg/ml, and particularly 1 mg/ml

to 5 mg/ml. That is, the amount of zymolase to be used is preferably 50 unit/ml to 500 unit/ml, particularly 100 unit/ml to 500 unit/ml.

Example 17

Examination of Optimal Condition (Titer) of Enzymatic Treatment

(1) Production of Digested sample

[1] Preparation of U937 cell

U937 cells (monocyte established cell line: ATCC CRL-1593.2) were cultured in an RPMI 1640 medium (25 ml) within a cell culture flask (175 cm^2) in a 5% carbon dioxide gas incubator at 37°C. Next, the U937 cell culture liquid was placed in a 50 ml centrifuge tube, and centrifuged at 4°C for 10 minutes at 220 × g to recover the U937 cells. Then, thus recovered U937 cells were suspended in 200 µl of PBS, and the cell number was counted with a counting chamber. The cell number was adjusted to 1×10^4 cells/µl to 2×10^4 cells/µl.

[2] Preparation of Bacterial Digested sample

Staphylococcus aureus (ATCC 12600), *Staphylococcus epidermidis* (ATCC 14990), *Pseudomonas aeruginosa*(ATCC 10145), *Enterococcus faecalis* (ATCC 19433) and *Escherichia coli* (ATCC 11775) were inoculated in each 5 ml of BHI culture medium, and cultured at 37°C for 8 hours or longer. The cultured bacterial liquid was centrifuged at 4°C for 10 minutes at 2,000×g to collect the bacteria. After discarding the supernatant, the bacterial pellet was suspended in 5 ml of PBS, and centrifugation was conducted once again at 4°C for 10 minutes at 2,000×g to collect the bacteria. Thus collected bacteria were suspended in 5 ml of PBS and thereafter, 15 ml of bacterial liquids was produced prepared by diluting in PBS to give the turbidity (O.D. = 600 nm) of the bacterial liquid, which was measured with a absorbance meter, of 0.01 to 0.03 for *Staphylococcus aureus*, 0.01 to 0.03 for *Staphylococcus epidermidis*, 0.02 to 0.03 for *Pseudomonas aeruginosa*, 0.01 to 0.03 for *Enterococcus faecalis*, 0.02 to 0.03 for *Escherichia coli*, respectively. Thus produced

bacterial liquid was transferred into a separate 175 cm^2 flask for culture, and left to stand still at room temperature for 30 minutes. Fifty ml of heparinized healthy human blood was collected, and thereto was added the reagent for separating hemocyte at a ratio of 4 : 1, and left to stand still at 37° C for 30 minutes to yield the leukocyte fraction. Thus obtained leukocyte fraction was adjusted to 50 ml with PBS. The supernatant in the culture flask (*supra*) was gently discarded, and each 10 ml of the leukocyte fraction diluted in PBS was added to the flask followed by leaving to stand still at room temperature for 10 minutes. The supernatant in the flask for culture was discarded, and the leukocytes attached to the bottom of the flask were recovered in a 15 ml centrifuge tube with 10 ml of PBS containing 0.02% EDTA, and centrifuged at 4° C for 10 minutes at 140×g to 180×g to collect the leukocytes. Because contamination of erythrocytes was found in the collected leukocytes, precipitates of the leukocytes were gently suspended in 1 ml of sterile purified water to allow hemolysis, subjected to isotonization through adding 14 ml of PBS, followed by centrifugation once again at 4° C for 10 minutes at 140 × g to 180×g to collect the leukocytes. The collected leukocytes were suspended in PBS, and cell number was counted with a counting chamber to adjust to give 1×10^4 cells/µl to 5×10^4 cells/µl. These digested samples were referred to as SA digested sample, SE digested sample, PA digested sample, EF digested sample and EK digested sample.

[3] Smear and Fixation

Each 5 µl of U937 cells prepared in Example 17 (1) [1] and each 5 µl of each bacterial digested sample produced in Example 17 (1) [2] were smeared on each well of the APS coated slide glass, and air dried. Next, after immersing the slide glass in the Carnoy s fixative described in Example 2 for 20 minutes, it was immersed in 75% ethanol for 5 minutes. After washing Carnoy' s fixativeand air drying, the slide glass was stored at 4° C until use in the test (see, Example 2). Next, pretreatment of the fixed sample was carried out according to Example 3.

(2) Standard and Process for Testing Digested sample

[1] Cell Number

Cell number to be smeared and fixed on the slide glass of each bacterial digested sample was 5.0×10^4 to 2.5×10^5 cells/well, whilst cell number of U937 cells was 5.0×10^4 to 1.0×10^5 cells/well.

[2] Phagocytosis Rate

The bacterial digested sample smeared and fixed on the slide glass was stained with an acridine orange staining solution, and about 200 cells were randomly counted with a fluorescence microscope ($\times$ 1,000). Among the measured cells, cells including bacteria phagocytized within the cells (cells with any change characteristicic in phagocytosis found in morphology, as shown by arrows in Fig. 6) were determined as positive cells, and the phagocytosis rate (%) was calculated according to the mathematical formula below.

Phagocytosis rate (%) = [(Positive cell number/Measured cell number)$\times$100]

Thus calculated phagocytosis rate (%) of each bacterial digested sample was 10% or greater.

[3] Test Process

The digested sample produced in Example 17 (2) [1] and [2] was employed as a specimen. The SA digested sample used had the phagocytosis rate of 23% with 1.98×10^5 cells/well. The SE digested sample had the phagocytosis rate of 27% with 1.74×10^5 cells/well. Moreover, the EF digested sample had the phagocytosis rate of 34% with 6.40×10^4 cells/well.

Using the slide glass having each digested sample smeared thereon, the enzymatic pretreatment was performed according to the process described in Example 3. Next, the slide glass after completing the enzymatic pretreatment was placed in a humid box, and the reaction was allowed by dropping 1 ml of each enzyme solution prepared to give each titer on the smeared site of the specimen. Thereafter, the slide glass was immersed in PBS

containing 0.2 mol/l hydrochloric acid, and in 70% ethanol respectively, for 10 minutes, and air dried. After immersing this slide glass in PBS containing 70 mmol/l sodium hydroxide for 3 minutes, and in 70% ethanol for 10 minutes, it was air dried and stained with 1% acridine orange staining solution. Then, evaluation was made with a fluorescence microscope (× 1,000). For *Staphylococcus aureus* and *Staphylococcus epidermidis*, examination of the optimal titer was conducted with lysostafin. In order to examine the optimal titer when N-acetylmuramidase and lysozyme are used in combination for *Enterococcus faecalis*, examination on optimal titer of lysozyme was conducted in cases where N-acetylmuramidase was fixed at 100 unit/ml, and on optimal titer of N-acetylmuramidase in cases where lysozyme was fixed at 10,000 unit/ml. The determination was made as adequate when no bacterial body was identified in the leukocytes by the enzymatic treatment.

[4] Results

For the lysis of *Staphylococcus aureus*, as described in Table 4, sufficient effects were exerted at the titer of lysostafin of 1 unit/ml, however, upon lysis of *Staphylococcus epidermidis*, the titer of lysostafin of 10 unit/ml or greater was necessary. Therefore, the optimal titer of lysostafin was set to be 10 unit/ml to 100 unit/ml. In addition, for the lysis of *Enterococcus faecalis*, lysis did not occur with the titer of N-acetylmuramidase of 10 unit/ml or less when the titer of lysozyme was fixed at 10,000 unit/ml. In respect of lysozyme, when the titer of N-acetylmuramidase was fixed at 100 unit/ml, lysis did not occur with the titer of lysozyme of 1,000 unit/ml or less, as described in Table 5. Therefore, the optimal titer of N-acetylmuramidase was set to be 100 unit/ml to 1,000 unit/ml, whilst the optimal titer of lysozyme was set to be 10,000 unit/ml to 100,000 unit/ml. The results are shown in Fig. 7. In the Figure, depicted are states of: (a) the digested sample of *Staphylococcus aureus* prior to the enzymatic treatment, (b) the digested sample of *Enterococcus faecalis* prior to the enzymatic treatment, (c) the sample (a) following the enzymatic treatment,

and (d) the sample (b) following the enzymatic treatment.

Table 4: Optimal Titer for Enzymatic Treatment of Lysostafin on *S. Aureus*, *S. epidermidis*

Lysostafin Titer (U/mL) / Digested Samples		0	0.1	1	10	100	1,000
SA Digested Sample	once	inadequate	Inadequate	adequate	adequate	adequate	adequate
	twice	inadequate	inadequate	adequate	adequate	adequate	adequate
	thrice	inadequate	inadequate	adequate	adequate	adequate	adequate
SE Digested Sample	once	inadequate	inadequate	inadequate	adequate	adequate	adequate
	twice	inadequate	inadequate	inadequate	adequate	adequate	adequate
	thrice	inadequate	inadequate	inadequate	adequate	adequate	adequate

Table 5: Optimal Titer of Enzymatic Treatment of N-acetylmuramidase and lysozyme on *E. faecalis*

N-acetyl muramidase titer (U/mL) / Digested Sample		0	1	10	100	1,000	10,000
EF Digested Sample	once	Inadequate	inadequate	inadequate	adequate	adequate	adequate
	twice	Inadequate	inadequate	inadequate	adequate	adequate	adequate
	thrice	Inadequate	inadequate	inadequate	adequate	adequate	adequate
Lysozyme titer (U/mL) / Digested Sample		0	10	100	1,000	10,000	100,000
EF Digested Sample	once	Inadequate	inadequate	inadequate	inadequate	adequate	adequate
	twice	Inadequate	inadequate	inadequate	inadequate	adequate	adequate
	thrice	Inadequate	inadequate	inadequate	inadequate	adequate	adequate

Applications of these results obtained using the digested samples to the present invention could result in similar results. Therefore, the optimal titer of each enzyme as described above in the identification of a causative microorganism of an infectious disease in the clinical specimen of the present invention was also set similarly.

Example 18

Examination on Optimal Condition of Enzymatic Treatment (Temperature)

Using a slide glass including each digested sample smeared thereon, examination was conducted according to the process described in example 17 (2) [3]. Time period of the enzymatic treatment in this test was 30 minutes, and the temperature for examination was 4°C, 25°C, 37°C, 42°C, and 60°C. Moreover,

titer of each enzyme was N-acetylmuramidase (100 unit/ml, manufactured by Seikagaku Corporation), lysozyme (10,000 unit/ml, manufactured by Seikagaku Corporation), lysostafin (10 unit/ml, manufactured by SIGMA).

Determination was conducted according to the process described in example 17 (2) [3]. As a consequence, for *Staphylococcus aureus*, no bacterial body was found in the leukocytes in the range of temperature of 4°C to 60°C. For *Staphylococcus epidermidis*, although bacterial bodies remained in the leukocytes at the temperature of 4°C and 25°C, no bacterial body was found at 37°C or higher. Further, for *Enterococcus faecalis*, although bacterial bodies remained at the temperature of treatment of 4°C, 25°C and 60°C, no bacterial body was found at 37°C and 42°C. Hence, the optimal temperature for the enzymatic treatment was set to be 37°C to 42°C. The results are shown in Table 6.

Table 6: Optimal Temperature for Treatment of Enzyme Reagent

Digested Samples \ Temperature for Treatment (℃)		4	25	37	42	60
SA Digested Sample	Once	adequate	adequate	adequate	adequate	adequate
	twice	adequate	adequate	adequate	adequate	adequate
	thrice	adequate	adequate	adequate	adequate	adequate
SE Digested Sample	once	inadequate	inadequate	adequate	adequate	adequate
	twice	inadequate	inadequate	adequate	adequate	adequate
	thrice	inadequate	inadequate	adequate	adequate	adequate
EF Digested Sample	once	inadequate	inadequate	adequate	adequate	inadequate
	twice	inadequate	inadequate	adequate	adequate	inadequate
	thrice	inadequate	inadequate	adequate	adequate	inadequate

Applications of these results obtained using the digested samples to the present invention could result in similar results. Therefore, the optimal temperature of the enzymatic treatment in the identification of a causative microorganism of an infectious disease in the clinical specimen of the present invention was also set similarly.

Example 19

Examination on Optimal Condition of Enzymatic Treatment (Time)

Digested samples produced according to the process described in Example 17 (1) [1] and [2] were used as specimens. Time period of the examination was 0 minute, 10 minutes, 20 minutes, 30 minutes, 60 minutes and 120 minutes. Phagocytosis rate of the used SA digested sample was 18% with 7.80×10^4 cells/well. Phagocytosis rate of the used SE digested sample was 34% with 1.10×10^5 cells/well. Further, phagocytosis rate of the EF digested sample was 28% with 1.30×10^5 cells/well.

Using the slide glass including each digested sample smeared thereon, examination was conducted according to the process described in example 17 (2) [3]. Temperature for the enzymatic treatment in this test was 37°C, and titer of each enzyme was 100 unit/ml for N-acetylmuramidase, 10,000 unit/ml for lysozyme, 10 unit/ml for lysostafin. Determination was conducted according to the process described in example 17 (2) [3]. As a consequence, for all of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* digested samples, no bacterial body was found in the leukocytes with the time period of the enzymatic treatment of 20 minutes or longer (inadequate at 0 minute and 10 minutes). Therefore, the optimal time period of the enzymatic treatment is at least 15 minutes or longer, preferably 20 minutes or longer, and still preferably 30 minutes to 60 minutes. The results are shown in Table 7.

Table 7: Optimal Time Period of Treatment of Enzyme Reagent

Digested Samples \ Time of enzyme-treatment (minutes)		0	10	20	30	60	120
SA Digested Sample	once	inadequate	inadequate	adequate	adequate	adequate	adequate
	twice	inadequate	inadequate	adequate	adequate	adequate	adequate
	thrice	inadequate	inadequate	adequate	adequate	adequate	adequate
SE Digested Sample	once	inadequate	inadequate	adequate	adequate	adequate	adequate
	twice	inadequate	inadequate	adequate	adequate	adequate	adequate
	thrice	inadequate	inadequate	adequate	adequate	adequate	adequate
EF Digested Sample	once	inadequate	inadequate	adequate	adequate	adequate	adequate
	twice	inadequate	inadequate	adequate	adequate	adequate	adequate
	thrice	inadequate	inadequate	adequate	adequate	adequate	adequate

Applications of these results obtained using the digested samples to the present invention could result in similar results. Therefore, the optimal time period of the enzymatic treatment in the identification of a causative microorganism of an infectious disease in the clinical specimen of the present invention was also set similarly.

Example 20

Examination on Optimal Condition of Enzymatic Treatment (Time)

In *in situ* hybridization reaction according to the present invention, concentration of the probe is an important factor which affects the hybridizing velocity. When the probe concentration is too low, the reaction velocity may be lowered, leading to the possibility of unclear signal. Furthermore, use of an excess amount of probe may result in grounds for nonspecific reaction.

Thus, optimal concentration was examined in connection with various probe solutions. First, the digested samples produced according to the process described in Example 17(1) [1] and [2] were used as specimens. The phagocytosis rate of the used SA digested sample was 24% with 1.48×10^5 cells/well. The phagocytosis rate of the SE digested sample was 28% with 2.07×10^5 cells/well. The phagocytosis rate of the PA digested sample was 11% with 1.59×10^5 cells/well. In addition, the phagocytosis rate of the EF digested sample was 24% with 1.72×10^5 cells/well. The phagocytosis rate of the EK digested sample was 12% with 1.63×10^5 cells/well. Using the slide glass including each digested sample smeared thereon, examination was conducted according to the process described in Example 17(2) [3]. The probes for use were labelled with digoxigenin, and the concentration of each probe for *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* was adjusted to 0.06 ng/μl, 0.6 ng/μl, 1.2 ng/μl, 1.8 ng/μl, 2.4 ng/μl, 3 ng/μl, respectively. The probe solution prepared to each concentration described above was used on the slide glass including the digested sample smeared thereon (see, Fig. 8), and examined according to the process described in Examples 3-11.

Consequently, the signal became unclear at lower concentration (0.06 ng/μl), and on the other hand, increase in background was observed at higher concentration (2.4 ng/μl and 3 ng/μl). Therefore, the concentrations of probes of SA, SE, PA, EF and EK were determined to be 0.6 to 1.8 ng/μl, preferably 0.6 to 1.2 ng/μl. Moreover, since an inadequate result was yielded at 0.06 ng/μl, while an adequate result was yielded at 0.6 ng/μl, it is preferably determined to be 0.1 ng/μl or greater.

Furthermore, since an inadequate result was yielded at 2.4 ng/μl, and an adequate result was yielded at 1.8 ng/μl, it is preferably determined to be 2.2 ng/μl or less. The results are shown in Tables 8-12 below.

Table 8: SA probe

Digested sample	Probe concentration (ng/µL)					
	0.06	0.6	1.2	1.8	2.4	3
SA digested sample	−	+	+	+	+	+
SE digested sample	−	−	−	−	+	+
PA digested sample	−	−	−	−	+	+
EF digested sample	−	−	−	−	+	+
EK digested sample	−	−	−	−	+	+

Table 9: SE probe

Digested sample	Probe concentration (ng/µL)					
	0.06	0.6	1.2	1.8	2.4	3
SA digested sample	−	−	−	−	−	+
SE digested sample	−	+	+	+	+	+
PA digested sample	−	−	−	−	−	+
EF digested sample	−	−	−	−	−	+
EK digested sample	−	−	−	−	−	+

Table 10: PA probe

Digested sample	Probe concentration (ng/µL)					
	0.06	0.6	1.2	1.8	2.4	3
SA digested sample	−	−	−	−	−	−
SE digested sample	−	−	−	−	+	+
PA digested sample	−	+	+	+	+	+
EF digested sample	−	−	−	−	−	+
EK digested sample	−	−	−	−	−	+

Table 11: EF probe

Digested sample	Probe concentration (ng/μL)					
	0.06	0.6	1.2	1.8	2.4	3
SA digested sample	−	−	−	−	−	+
SE digested sample	−	−	−	−	+	+
PA digested sample	−	−	−	−	+	+
EF digested sample	−	+	+	+	+	+
EK digested sample	−	−	−	−	−	−

Table 12: EK probe

Digested sample	Probe concentration (ng/μL)					
	0.06	0.6	1.2	1.8	2.4	3
SA digested sample	−	−	−	−	+	+
SE digested sample	−	−	−	−	+	+
PA digested sample	−	−	−	−	+	+
EF digested sample	−	−	−	−	+	+
EK digested sample	−	+	+	+	+	+

Applications of these results obtained using the digested samples to the present invention could result in similar results. Therefore, the optimal concentration of each probe described above in the identification of a causative microorganism of an infectious disease in the clinical specimen of the present invention was also set similarly.

Example 21

Examination on Hybridization Temperature

Reaction temperature in the hybridization reaction is a parameter which affects the hybridizing velocity and stability of the hybrid. Because high temperature of the hybridization reaction is known to deteriorate the cell morphology, examination of the optimal temperature (4°C, 25°C, 37°C, 42°C,

50° C and 60° C) was performed.

First, the digested samples produced according to the process described in Example 17(1) [1] and [2] were used as specimens. The phagocytosis rate of the used SA digested sample was 31% with 1.38×10^5 cells/well. The phagocytosis rate of the SE digested sample was 42% with 1.95×10^5 cells/well. The phagocytosis rate of the PA digested sample was 14% with 1.27×10^5 cells/well. In addition, the phagocytosis rate of the EF digested sample was 48% with 1.05×10^5 cells/well. The phagocytosis rate of the EK digested sample was 17% with 1.85×10^5 cells/well.

Using the slide glass including the digested sample and U937 cells smeared and fixed thereon (see, Fig. 9), examination was conducted according to the process described in Examples 3-11. Consequently, no stable signal was observed for each type of probe at the hybridization temperature of 4° C or less owing to the lowered hybridization velocity. Further, at 60° C, changes in cell morphology were detected, and thus no stable signal was observed. In addition, at 25° C and 50° C, detection could be executed better compared to at the temperature of 37° C and 42° C, although the signal was unclear. Hence, optimal temperature of the hybridization may be 25° C to 50° C, more preferably 37 to 42° C. The results are shown in Tables 13-17 below.

Table 13: SA probe

Digested sample	Hybridization temperature (℃)					
	4	25	37	42	50	60
SA digested sample	−	+	+	+	+	+
SE digested sample	−	−	−	−	−	−
PA digested sample	−	−	−	−	−	−
EF digested sample	−	−	−	−	−	−
EK digested sample	−	−	−	−	−	−

Table 14: SE probe

Digested sample	Hybridization temperature (℃)					
	4	25	37	42	50	60
SA digested sample	—	—	—	—	—	—
SE digested sample	+	+	+	+	+	—
PA digested sample	—	—	—	—	—	—
EF digested sample	—	—	—	—	—	—
EK digested sample	—	—	—	—	—	—

Table 15: PA probe

Digested sample	Hybridization temperature (℃)					
	4	25	37	42	50	60
SA digested sample	—	—	—	—	—	—
SE digested sample	—	—	—	—	—	—
PA digested sample	—	+	+	+	+	—
EF digested sample	—	—	—	—	—	—
EK digested sample	—	—	—	—	—	—

Table 16: EF probe

Digested sample	Hybridization temperature (℃)					
	4	25	37	42	50	60
SA digested sample	—	—	—	—	—	—
SE digested sample	—	—	—	—	—	—
PA digested sample	—	—	—	—	—	—
EF digested sample	+	+	+	+	+	—
EK digested sample	—	—	—	—	—	—

Table 17: EK probe

Digested sample	Hybridization temperature (℃)					
	4	25	37	42	50	60
SA digested sample	—	—	—	—	—	—
SE digested sample	—	—	—	—	—	—
PA digested sample	—	—	—	—	—	—
EF digested sample	—	—	—	—	—	—
EK digested sample	—	+	+	+	+	—

Applications of these results obtained using the digested samples to the present invention could result in similar results. Therefore, the optimal temperature of hybridization in the identification of a causative microorganism of an infectious disease in the clinical specimen of the present invention was also set similarly.

Example 22

Examination on Hybridization Time Period

The digested samples produced according to the process described in Example 17(1) [1] and [2] were used as specimens, and examination was conducted on the time period of hybridization of 10 minutes, 60 minutes, 90 minutes, 120 minutes, 180 minutes and 900 minutes. The phagocytosis rate of the used SA digested sample was 47% with 1.45×10^5 cells/well. The phagocytosis rate of the SE digested sample was 47% with 1.33×10^5 cells/well. The phagocytosis rate of the PA digested sample was 15% with 1.91×10^5 cells/well. In addition, the phagocytosis rate of the EF digested sample was 41% with 1.45×10^5 cells/well. The phagocytosis rate of the EK digested sample was 20% with 1.23×10^5 cells/well.

Using the slide glass including the digested sample and U937 cells smeared and fixed thereon (same as one shown in Fig. 9), examination was conducted according to the process described in Examples 3-11. Consequently, although no signal was observed

with the time period of hybridization of 10 minutes, a signal was observed at 60 minutes or greater, and a stable signal was observed at 90 minutes or greater. Further, no alteration in detection of the signal was observed also with the time period of hybridization of 900 minutes. Therefore, it is preferred that the time period is at least 30 minutes or greater, preferably 60 minutes or greater, and more preferably 90 minutes or greater. More preferred optimal time period of hybridization may be set to be 120 minutes to 900 minutes. The results are shown in Tables 18-22 below.

Table 18: SA probe

Digested sample	Hybridization time (minutes)					
	10	60	90	120	180	900
SA digested sample	−	+	+	+	+	+
SE digested sample	−	−	−	−	−	−
PA digested sample	−	−	−	−	−	−
EF digested sample	−	−	−	−	−	−
EK digested sample	−	−	−	−	−	−

Table 19: SE probe

Digested sample	Hybridization time (minutes)					
	10	60	90	120	180	900
SA digested sample	−	−	−	−	−	−
SE digested sample	+	+	+	+	+	+
PA digested sample	−	−	−	−	−	−
EF digested sample	−	−	−	−	−	−
EK digested sample	−	−	−	−	−	−

Table 20: SE probe

Digested sample	Hybridization time (minutes)					
	10	60	90	120	180	900
SA digested sample	–	–	–	–	–	–
SE digested sample	–	–	–	–	–	–
PA digested sample	–	+	+	+	+	+
EF digested sample	–	–	–	–	–	–
EK digested sample	–	–	–	–	–	–

Table 21: EF probe

Digested sample	Hybridization time (minutes)					
	10	60	90	120	180	900
SA digested sample	–	–	–	–	–	–
SE digested sample	–	–	–	–	–	–
PA digested sample	–	–	–	–	–	–
EF digested sample	+	+	+	+	+	+
EK digested sample	–	–	–	–	–	–

Table 22: EK probe

Digested sample	Hybridization time (minutes)					
	10	60	90	120	180	900
SA digested sample	–	–	–	–	–	–
SE digested sample	–	–	–	–	–	–
PA digested sample	–	–	–	–	–	–
EF digested sample	–	–	–	–	–	–
EK digested sample	–	+	+	+	+	+

Applications of these results obtained using the digested samples to the present invention could result in similar results.

Therefore, the optimal time period of hybridization in the identification of a causative microorganism of an infectious disease in the clinical specimen of the present invention was also set similarly.

Example 23

Influence of Surfactant Added to Hybridization Solution

The digested samples produced according to the process described in Example 17(1) [1] and [2] were used as specimens. When any of various surfactants (SDS, lauryl sarcosine, saponin, BRIJ35, Tween 20, Triton X-100) was added to the probe dilution solution followed by hybridization carried out according to Example 7, the detection sensitivity was dramatically enhanced by adding 0.25% SDS. In addition, the detection sensitivity could be improved by lauryl sarcosine, BRIJ 35 or Tween 20. The results are shown in Table 23 below.

Table 23

Surfactant	Signal detection
None added	+
SDS	+++
Lauryl sarcosine	++
Saponin	+
BRIJ 35	++
Tween 20	++
Triton X-100	+

Furthermore, as a consequence of using SDS at various concentrations, it was revealed that preferable concentration was 1% or less, more preferably 0.1% to 0.5%, and still more preferably 0.25%.

Applications of these results obtained using the digested samples to the present invention could result in similar results.

Therefore, also in the present invention, it is preferred that a surfactant, particularly SDS, is added at the step of *in situ* hybridization.

Example 24

Examination on Chain Length of Probe Used in Hybridization

Staphylococcus aureus probe (SA-24 (SEQ ID NO: 1)) and *Pseudomonas aeruginosa* probe (P2-2 (SEQ ID NO: 7)) were labelled with digoxigenin.

First, 1 µg of purified each type of the DNA probe was prepared to give the total volume of 50 µg with 5 µl of 10× L.B. (0.5 mol/l Tris-hydrochloric acid (pH 7.5), 50 mmol/l magnesium chloride, 5 µl of 0.5 mg bovine serum albumin), 5 µl of 100 mmol/l dithiothreitol, each 1 nmol of dNTPs (A, G, C), 0.5 nmol of digoxigenin-dUTP (Dig-dUTP), each 0.5 nmol of dTTP, 3 µl of DNase (amount corresponding to 25 mU, 75 mU and 200 mU), 1 µl of 10 U/µl DNA polymerase and an appropriate amount of sterile purified water. Digoxigenin labelling was performed at 15° C for 2 hours. After the labelling, the mixture was boiled for 5 minutes to terminate the reaction. The reaction liquid after the termination was injected into a spin column (CENTRI-SEP COLUMUNS CS901, PRINCETON SEPARATIONS, INC.), and centrifuged at 25° C for 2 minutes (3,000×g) to remove free nucleotides. Then, concentration of the eluate was measured by an absorbance meter. Electrophoresis was performed on a 3% agarose gel to confirm the size.

Next, DNA in the agarose gel was transferred to a nitrocellulose membrane by Southern blotting method. Then, the membrane was immersed in 2% blocking reagent (manufactured by Roche) for 30 minutes, and thereafter, alkaline phosphatase labelled anti-digoxigenin antibody in an amount of 1/5,000 was added thereto and the immersion was allowed for 30 minutes. Next, the membrane was washed twice by shaking in 100 mmol/l Tris-hydrochloric acid (pH 7.5) and 150 mmol/l sodium chloride for 10 minutes. Thereafter, washing was executed by shaking in

100 mmol/l Tris-hydrochloric acid (pH9.5) and 150 mmol/l sodium chloride for 10 minutes. Then, color development was conducted by immersing in an NBT/BCIP solution.

Finally, the membrane was immersed in purified water to stop the color development, and dried. Consequently, as shown in Fig. 10 for (a) use of the SA probe and (b) use of the PA probe, respectively, it was indicates that in cases where cleavage was conducted using 25 mU of DNase (in Figure, lane 1) such that the chain length distributes the base length of predominantly about 350 to about 600, high labelling efficiency was achieved. When thus resulting probe for detection was used in the process for detecting a causative microorganism of an infectious disease according to the present invention in which a digested sample or a clinical specimen from a patient suffering from an infectious disease was used to carry out hybridization, a signal could be detected with excellent sensitivity. Therefore, it was reveled that chain length of the probe used in the hybridization may be the base length of about 350 to about 600, and preferably the base length of about 350 to about 550.

Example 25

Examination on Probe used in Hybridization

Escherichia coli digested samples produced according to the process described in Example 17(1) [1] and [2] were used as specimens to examine on the probes for detection.

Probes for detection were prepared through labelling with digoxigenin as described in Example 24 from EC-24 (SEQ ID NO: 11), EC-34 (SEQ ID NO: 12) and EC-39 (SEQ ID NO: 13) such that they have the base length of about 350 to about 600, and used alone or in combination of those three, respectively. From thus obtained results, it was evident that the signal could be detected more clearly resulting in elevated sensitivity for (d) the mixed probe MIX prepared by mixing the three ((a) EC-24, (b) EC-34 and (c) EC-39), than for each (a) EC-24, (b) EC-34 or (c) EC-39 used alone, as shown in Fig. 11.

INDUSTRIAL APPLICABILITY

Since the *in situ* hybridization according to the present method can offer the stable signals within two hours or less, detection results can be presented very rapidly. Obviously, such rapid detection demonstrates the value of the present method in the rapid diagnosis of sepsis.

CLAIMS

1. A method for detecting and/or identifying causative microorganisms of infectious diseases by taking phagocytes from the clinical specimens containing active phagocytes, immobilizing the phagocytes so taken, treating the phagocytes to improve cell membrane permeabilities thereof, further treating the phagocytes to bare DNA in the causative microorganisms which might be existed in the phagocytes, *in situ* hybridizing DNA so bared with detective DNA probe(s) which can hybridize with such bared DNA under stringent conditions, and detecting and/or identifying the causative microorganisms based on signals so detected, the method comprises at least one condition(s) to be selected from the following conditions (1)-(8) of;

(1) Cell density (**X** cells/ml) of the phagocytes to be immobilized is 5×10^{6} cells/ml$<$ **X** cells/ml$<1\times10^{8}$ cells/ml,

(2) Lysostaphin is applied into the step to bare DNA in the titer of from 1 Unit/ml to 1,000 Units/ml,

(3) Lysozyme is applied into the step to bare DNA in the titer of from 1,000 Units/ml to 1,000,000 Units/ml,

(4) N-acetylmuramidase is applied into the step to bare DNA in the titer of from 10 Units/ml to 10,000 Units/ml,

(5) Zymolyase is applied into the step to bare DNA in the titer of from 50 Units/ml to 500 Units/ml,

(6) Surfactant is applied into the step of *in situ* hybridization,

(7) Such DNA probe(s) is/are one or more DNA probe(s) to be determined with their chain length of from 350 bases to 600 bases, and

(8) Concentration of such DNA probe(s) is from 0.1 ng/μl to 2.2 ng/μl.

2. The method according to Claim 1, wherein said step to bare DNA employs one or more enzyme(s) selected from Lysostaphin in the titer of 10-100 Units/ml, Lysozyme in the titer of 10,000-100,000 Units/ml, N-acetylmuramidase in the titer of 100-1,000 Units/ml and Zymolyase in the titer of 100-500 Units/ml.

3. The method according to Claim 1 or 2, wherein said step to bare DNA employs enzyme(s), and the enzyme(s) is/are subjected to a reaction to be performed under the temperature of 26℃-59℃ for 15-120 minutes.

4. The method according to any of Claims 1-3, wherein said step to bare DNA further employs subtance(s) to keep a form of the phagocytes.

5. The method according to Claim 4, wherein said subtance to keep a form of the phagocytes is phenylmethylsulfonyl fluoride.

6. The method according to Claim 5, wherein concentration of said phenylmethylsulfonyl fluoride is 10μmol/l～10mmol/l.

7. The method according to any of Claims 4-6, wherein said subtance to keep a form of the phagocytes is a substance dissolved into dimethylsulfoxide.

8. The method according to Claim 7, wherein said subtance to keep a form of the phagocytes is a substance dissolved in dimethylsulfoxide, and concentration of the dimethylsulfoxide in a solution to be employed in said step to bare DNA is adjusted to less than 5%.

9. The method according to any of Claims 1-8, wherein said *in situ* hybridization step is perfomed by hybridizing DNA with DNA probe(s) under the precence of surfactant(s).

10. The method according to Claim 9, wherein said surfactant is an anionic surfactant.

11. The method according to Claim 10, wherein said anionic surfactant is sodium dodecyl sulfate.

12. The method according to any of Claims 1-11, wherein a hybridization reaction in said *in situ* hybridization step is performed under the temperature of 25℃-50℃ for 30-900 minutes.

13. The method according to any of Claims 1-12, wherein said method comrises, prior to said immobilization step, a step to mount the phagocytes so taken onto the solid support, and the solid support is a slide coated with 3-aminopropyl triethoxysilane.

14. The method according to any of Claims 1-13, wherein the method employs, at said signal detection, a pigment to distinguish contrast between signals and cells/.

15. The method according to any of Claims 1-14, wherein said clinical specimen is blood.

16. A kit for detecting and/or identifying causative microorganisms of infectious diseases by taking phagocytes from the clinical specimens containing active phagocytes, immobilizing the phagocytes so taken, treating the phagocytes to improve cell membrane permeabilities thereof, further treating the phagocytes to bare DNA in the causative microorganisms which might be existed in the phagocytes, *in situ* hybridizing DNA so bared with detective DNA probe(s) which can hybridize with such bared DNA under stringent conditions, and detecting and/or identifying the causative microorganisms based on signals so detected, the kit comprises the following elements (1)-(2) of;

(1) at least one enzyme(s) to be employed in said step to bare DNA which is/are selected from Lysostaphin, Lysozyme, N-acetylmuramidase and Zymolyase; and

(2) at least one detective DNA probe(s).

17. A method for monitoring a gene of exogenous microorganisms digested with the phagocytes in the clinical specimens containing active phagocytes comprising the step of detecting the gene with *in situ* hybridization method employed in the method according to any of Claims 1-15, wherein the gene of exogenous microorganisms in the clinical specimens is monitored.

18. A method for diagnosing sepsis or bacteriemia comprising the step of identifying a gene of candidate causative microorganisms with *in situ* hybridization method employed in the method according to any of Claims 1-15, wherein the causative microorganisms for sepsis or bacteriemia are determined based on the identification results.

S E Q U E N C E L I S T I N G

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TSUNEYA OHNO

<120> Improved Method for Detecting and Identifying Causal Microorganisms of Infectious Diseases

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<151> 2001-05-31

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<220>
<223> Designated as SA-36

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<213> Staphylococcus epidermidis

<220>
<223> Designated as SE-3

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<212> DNA
<213> Staphylococcus epidermidis

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<223> Designated as SE-32

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<213> Pseudomonas aeruginosa

<220>
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<220>
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<211> 2441
<212> DNA
<213> Enterococcus faecalis

<220>
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<211> 3480
<212> DNA
<213> Enterococcus faecalis

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<213> Escherichia coli

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Application number / numéro de demande: JP02/05107

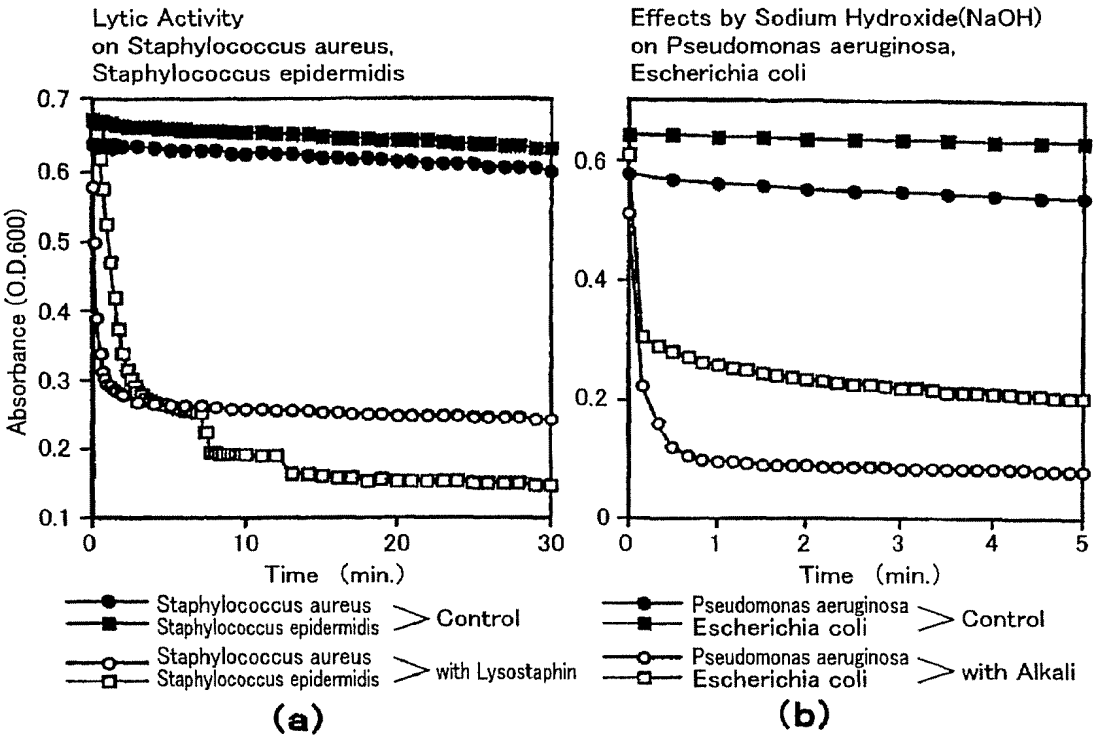
Figures:

Pages: 1/2/5/6/7/10/11

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)

FIGURE 3


Lytic Activity
on Staphylococcus aureus,
Staphylococcus epidermidis
Absorbance (O.D.600)
Time (min.)
Staphylococcus aureus
Staphylococcus epidermidis
Control
Staphylococcus aureus
Staphylococcus epidermidis
with Lysostaphin
(a)
Effects by Sodium Hydroxide(NaOH)
on Pseudomonas aeruginosa,
Escherichia coli
Time (min.)
Pseudomonas aeruginosa
Escherichia coli
Control
Pseudomonas aeruginosa
Escherichia coli
with Alkali
(b)

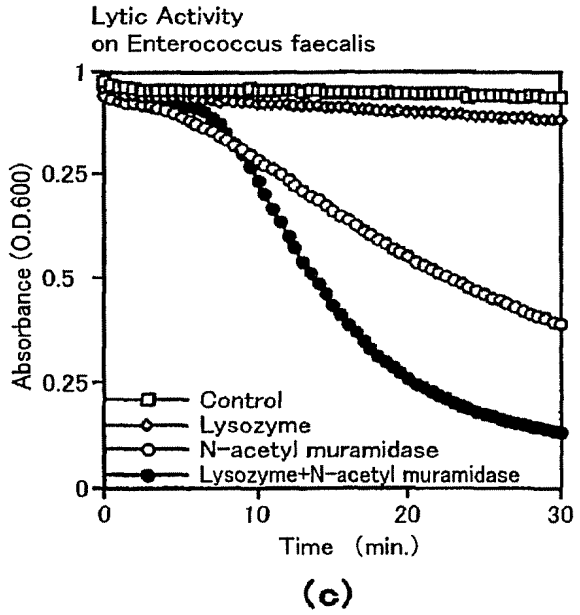

Lytic Activity
on Enterococcus faecalis
Absorbance (O.D.600)
Control
Lysozyme
N-acetyl muramidase
Lysozyme+N-acetyl muramidase
Time (min.)
(c)

FIGURE 4

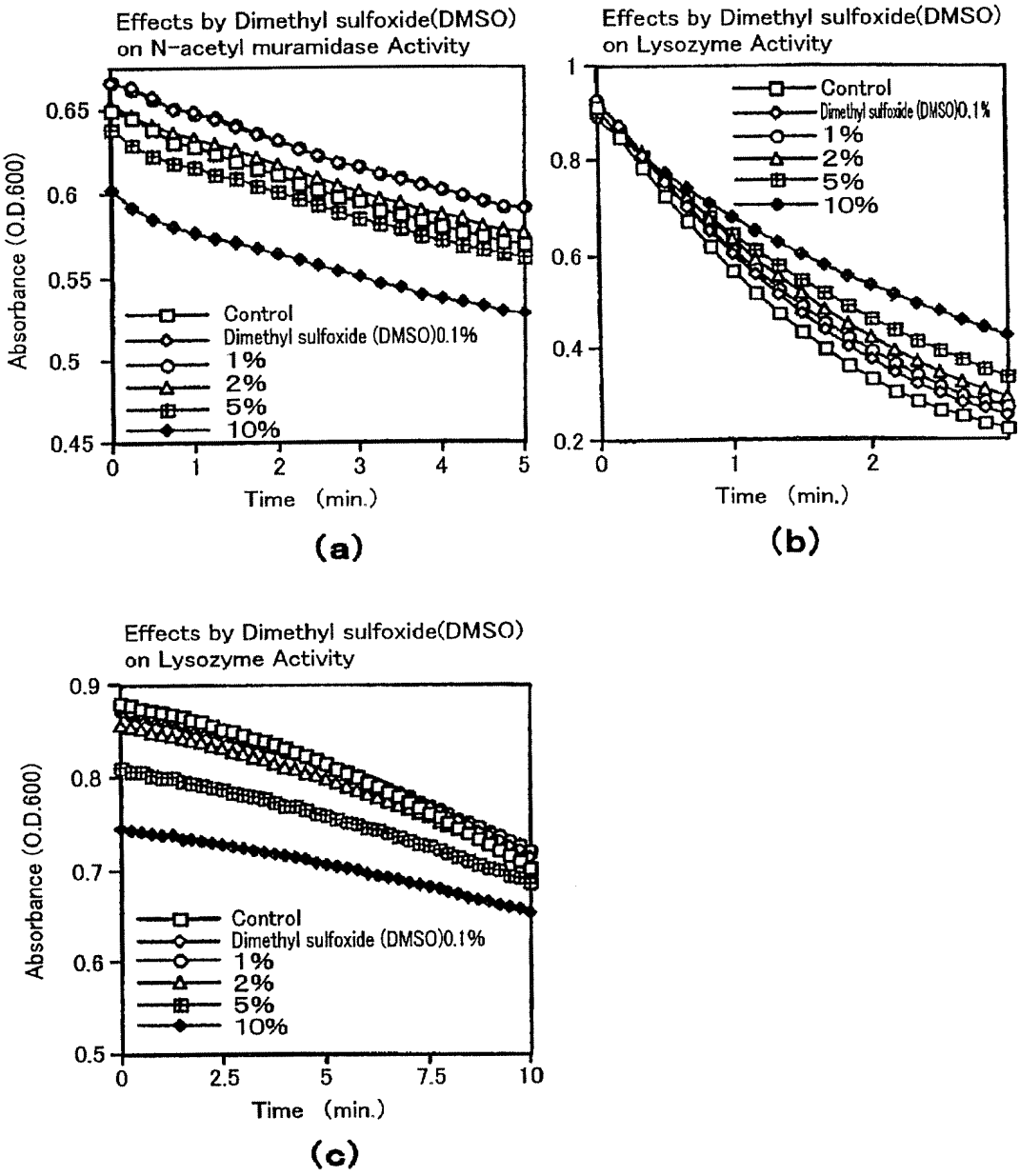
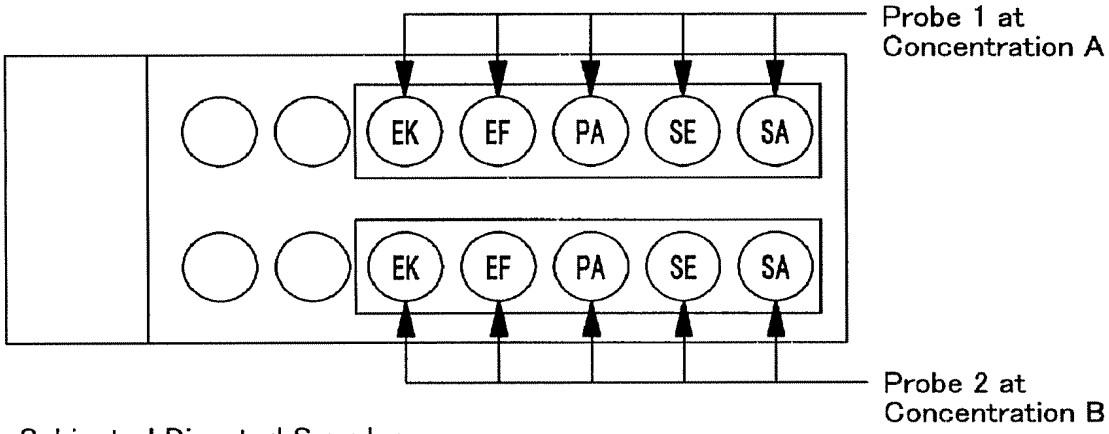

Effects by Dimethyl sulfoxide(DMSO)
on N-acetyl muramidase Activity
Absorbance (O.D.600)
Time (min.)
Control
Dimethyl sulfoxide (DMSO)0.1%
1%
2%
5%
10%
(a)
Effects by Dimethyl sulfoxide(DMSO)
on Lysozyme Activity
Time (min.)
Control
Dimethyl sulfoxide (DMSO)0.1%
1%
2%
5%
10%
(b)
Effects by Dimethyl sulfoxide(DMSO)
on Lysozyme Activity
Absorbance (O.D.600)
Time (min.)
Control
Dimethyl sulfoxide (DMSO)0.1%
1%
2%
5%
10%
(c)

FIGURE 8



Subjected Digested Samples

SA : SA Digested Sample

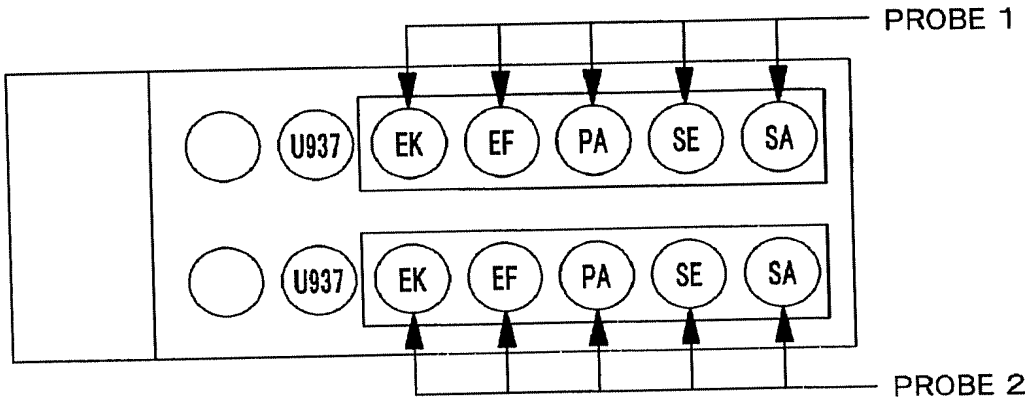
SE : SE Digested Sample

PA : PA Digested Sample

EF : EF Digested Sample

EK : EK Digested Sample

FIGURE 9



SA : SA Digested Sample
SE : SE Digested Sample
PA : PA Digested Sample
EF : EF Digested Sample
EK : EK Digested Sample